The Hoechst Centre for Basic Research

Pharmaceutical research in India may be broadly classified into innovational research, formulation and product development and process research. Innovational or basic research is still in its infancy in India. Expenditure on research and development, which is costly and risky, is a long term investment which may not be recouped for years. Companies cannot risk such investment if they cannot foresee reasonable returns. They must have an assurance that the enormous expenditure incurred would be capable of recovery. They must have a right to market the fruits of their research and must also have the confidence that the fruits of their expenditure and activities will be adequately protected.

As a part of their modest contribution to India's national efforts to make the country self-reliant in the sphere of basic research in the pharmaceutical industry and in keeping with its policy of laying great stress on Basic and Applied Research in its growth-oriented programme of development Hoechst set up a Centre for Basic Research at Mulund, near Bombay in 1972. Built at a cost of Rs. 13.9 mio it houses the Departments of Chemistry, Pharmacology and Microbiology and is manned by a staff of 120 which includes highly qualified, experienced and dedicated scientists many of whom hold doctoral degrees from foreign Universities.

In the last 8 years applications for 40 patents have been filed and 40 scientific papers have been presented at International Congresses and published in International scientific journals.

It is necessary that no research laboratory works in isolation. Accordingly, the Research Centre in Bombay works in close collaboration with other Hoechst research laboratories in a mutual exchange of scientific expertise. Two compounds are currently under development not only for India but also for Hoechst AG's world-wide activities.

Hoechst has already spent Rs. 90.0 mio on Research and Development in the past 6 years. Annual expenditure is over Rs. 12 mio.

Unpredictable though the monetary returns may be Hoechst Pharmaceuticals Limited's decision to enter the risky field of Basic Research proceeds from the fact that Hoechst believes in investing in the future of India, and in the health of her people.

This b[illegible] Amonkar

INTERNATIONAL SYMPOSIUM ON ANTIBIOTICS

Proceedings of the Second International Symposium
on Antibiotics organised by Hoechst Pharmaceuticals
Limited at Bombay, India, on the 14th of January 1980

Edited by Dr. J. Reden
Dr. B. N. Ganguli
Mr. A. A. Borges

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PREFACE

The Second International Symposium on Antibiotics organised by Hoechst Pharmaceuticals Limited, Bombay, India, was held at the Oberoi Intercontinental Hotel on January the 14th, 1980.

As early as 1972, when the Hoechst Centre for Basic Research was set up, it was recognised that such International Meetings, where scientists of repute can meet and exchange ideas and information, are essential for a vigorous and dynamic research programme.

Therefore, the first Symposium was held in 1976 and the second in 1980.

The second Symposium assumed particular importance because the nineteen seventies were very significant years in antibiotic research. They saw the emergence of several novel β-lactam antibiotics. The first break through was achieved with the discovery of the Cephamycins. This was followed by the discovery of the novel mono-cyclic β-lactams, the Nocardicins. Then came a series of β-lactam compounds which proved to be powerful inhibitors of the β-lactamases. Among these, Clavulanic acid and Thienamycin were the first to be identified. The recognition of these novel structures triggered off tremendous research activity in the field of semi-synthetic and synthetic chemistry.

A number of potent broad spectrum antibiotics were reported such as Cefotaxime, Ceftizoxime (FK 749), S-6059 etc. which were significantly more active than the classical β-lactams against resistant strains of micro-organisms.

In this Symposium, an attempt was made to present the latest information on this major field of development in β-lactam antibiotics. In addition, there were papers on the genetics of antibiotic producing micro-organisms and new ways to new antibiotics and secondary metabolites. Details of these papers have been presented in this brochure.

A panel discussion was held at the end of the Symposium in order to analyse and discuss the papers presented. The audience was invited to actively participate in the discussion. A wide variety of topics, ranging from the present state of chemotherapy in India to the evolution of micro-organisms, was debated.

Mr. R. Bink, Managing Director, Hoechst Pharmaceuticals Limited, Bombay, welcomed the guests and inaugurated the Symposium. Present on the occasion were Mr. Vittal Mallya, Chairman of the Board of Directors, HPL, Dr. H. G. Gareis, Head of the Pharmaceutical Division of Hoechst AG, Frankfurt, and Mr. D. Laengenfelder, Head of Pharmaceutical Sales, Hoechst AG, Frankfurt.

In addition to a number of foreign delegates approximately two hundred invitees from all over India comprising eminent scientists from National Laboratories, members of the Pharmaceutical Industry and the medical profession participated in the symposium.

On behalf of Hoechst Pharmaceuticals Limited, Bombay, India, we would like to thank all the participants and organisers for making the meeting a success.

J. Reden
B. N. Ganguli

SIDDIQI
REDEN

SCHRINNER
RICHMOND

FACING PAGE (TOP)

Dr. J. Coombes, Head, Milton Keynes Research Laboratories, Hoechst U.K.; Prof. O. Siddiqi, Head, Molecular Biology Group, Tata Institute of Fundamental Research, Bombay; Dr. J. Reden, Head, Research and Medical Development, Hoechst Pharmaceuticals Limited, Bombay who chaired the morning session.

(CENTRE)

Distinguished members of the audience in the front row.

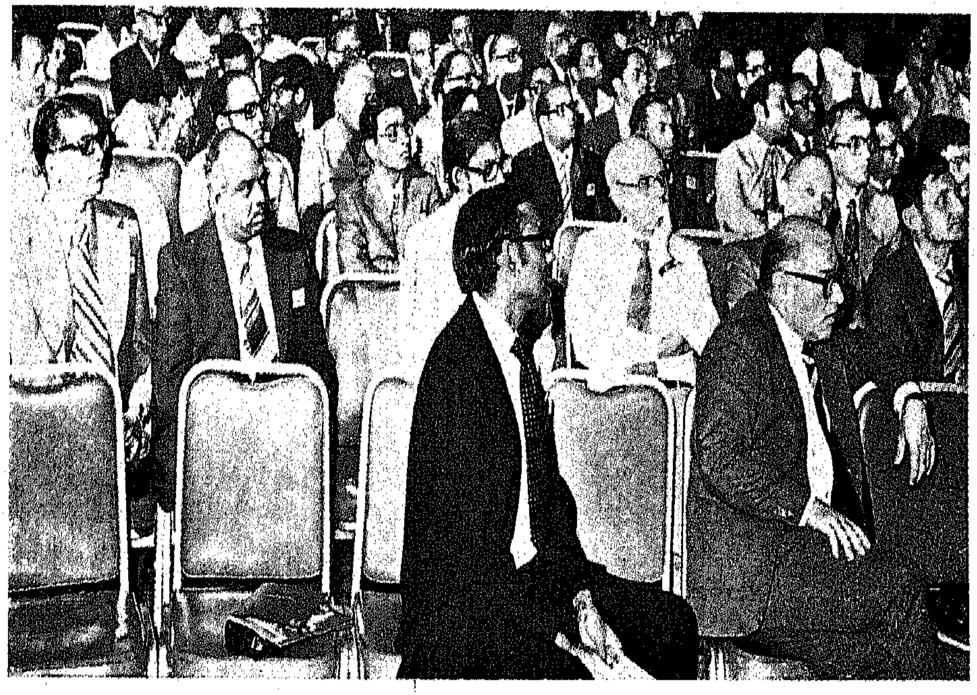
From left: Dr. H. G. Gareis, Head, Pharmaceuticals Division, Hoechst AG., Frankfurt; Prof. Dr. E von Wasielewski, Head, Pharmaceuticals Research, Hoechst AG., Frankfurt, Mr. D. Laengenfelder, Head, Pharmaceuticals Sales, Hoechst AG., Frankfurt; Mr. Vittal Mallya, Chairman, Hoechst Pharmaceuticals Limited, Bombay; Mr. R. Bink, Managing Director, Hoechst Pharmaceuticals Limited, Bombay.

(BOTTOM)

Dr. E. Schrinner, Head, Pharma Research, Chemotherapy, Hoechst AG., Frankfurt; Prof. M. Richmond, Prof. & Head of Bacteriology, University of Bristol, U.K.; Dr. P. V. Divekar, Manager, Microbiology Department, Hoechst Pharmaceuticals Limited, Bombay who chaired the afternoon session.

THIS PAGE

The two photographs on this page depict the keen interest of the delegates who participated in the proceedings of the Symposium.



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INTERNATIONAL SYMPOSIUM ON ANTIBIOTICS

Welcome address by Mr. R. Bink, Managing Director, Hoechst Pharmaceuticals Limited, Bombay.

Ladies and Gentlemen,

On behalf of the Board and Management of Hoechst Pharmaceuticals Limited, I welcome all of you to this Scientific Symposium on Antibiotics. It is most gratifying to see so many distinguished visitors, both from India and abroad, assembled in this room.

Our sincere thanks are due to Mr. Vittal Mallya, Chairman of our Board, Dr. H. Gareis, Head of Pharmaceuticals Division of Hoechst, and Mr. D. Laengenfelder, a former Managing Director of HPL and now Head of the Pharmaceuticals Sales Department, for taking precious time to participate in our Symposium.

I have the pleasure to extend our cordial welcome to the main speakers of today's programme: Prof. Zaehner from the University of Tuebingen, West Germany; Prof. Richmond, Bristol University, U.K.; Prof. Siddiqi from the Tata Institute of Fundamental Research, Bombay; Dr. Coombes, Hoechst Pharmaceuticals Research Laboratories, U.K.; Dr. Schrinner, Hoechst AG., Frankfurt, and Prof. v. Wasielewski, Director of Pharmaceutical Research, Hoechst AG, Frankfurt, who will be the Chairman of our concluding panel discussion.

We are happy to have so many renowned scientists and experts present here, as well as distinguished guests and representatives of the various establishments of Research and Industry and the Press.

Ladies and Gentlemen, allow me some comments on drug research and development, beginning with the question: Do we need new drugs?

Any doctor or hospital can easily provide the answer. There is no area of medicine that does not suffer due to inadequate drugs. Schizophrenics, arthritics and cancer patients can expect little from our present therapies.

Amoebiasis, which is so rampant in India, still needs a drug which is safe and effective. In the area of malaria and leprosy, more and more need for new drugs is being felt, as resistance to the older drugs is now occurring in a larger percentage of patients.

There should be little disagreement, therefore, about society's need for new and better drugs. But how do we find them? Discovery is related to available knowledge, to the adequacy of current methodology, to support of both basic and applied research, and to the ease with which discoveries can be turned into approved new remedies.

As far as *support of research* is concerned, it has to be mentioned that most applied research is performed by Industry. New drugs have come and will continue to come primarily from the larger innovative drug firms. Basic research in drug development is a challenging and complex task entailing the interaction of several scientific disciplines and calls for much investment in men and material. Hoechst has never hesitated to do these investments and has established *Research Centres*, besides Germany, in the United States, Japan, England, Egypt, Brazil and eight years ago in India as well. We are proud enough to state that in our Research Centre in Mulund, under the guidance of Dr. Dornauer, who recently went back to Germany, two very promising compounds have been discovered, and are now under development.

The amount of $260 Mio, spent by Hoechst in 1978 for Research & Development in the Pharmaceutical field alone, gives convincing evidence that Hoechst believes in research.

In the long run, only well-organised and successful research efforts will give the firm a basis for a prosperous future and, finally, will be for the benefit of mankind.

As mentioned before, basic research is followed by the second step in which discoveries are to be turned into approved remedies: the *drug development*. As you all know, nowadays this is one of the crucial points for research-oriented drug firms. During the last decades the development of a compound became more and more complicated, more time-consuming and extremely costly.

Taking into consideration how long it takes to develop a compound from its synthesis via biological and toxicological investigations and clinical trials to its introduction in the market (nowadays 8-10 years are the average), it becomes evident that a *life-time of patents* of 7 years in India is meaningless. Today, it takes a company about 50 million dollars to bring one of its new drugs into the market, if one considers the total costs of research programmes, that is the cost of working up the failures as well as the successes. Instead of encouraging the Pharmaceutical Industry to carry out drug research and to develop new remedies, the authorities in many countries build up new hurdles and more and more regulations.

It is self understood that pre-clinical, experimental and sufficient toxicological studies are necessary. However, some countries, for example, still demand repetitions of toxicological investigations of compounds which represent waste of animals, material, time and money. The increasing support of *generics*, given under the assumption that generics are cheaper than trademark-products in many countries, will create an additional setback in respect of the cost benefit ratio for innovative drug firms.

Since the cost of a trademarked product often embodies the immense research expenditure of the innovator, any short-term saving by generic prescribing has to be weighed against the ultimate effect—decreased innovative research.

Many countries have introduced price regulations with the objective of ensuring that drugs are supplied to the people at a reasonable price. Sometimes, these regulations are rather unrealistic with the result that the companies' profitability goes down and investment in research has to be curtailed.

Due to these rising problems, offensive research is going to be narrowed down increasingly into a defensive research, or in other words, the increase in cost of development will lead to a more cautious, less venturesome approach towards drug research.

Coming to an end, I should be allowed to mention that tomorrow Hoechst will launch in Germany a new antibiotic of the cephalosporin class, called Cefotaxime, a result of the joint research of Hoechst and Roussel, which shows most exciting properties and which was called by experts as the beginning of a new generation of cephalosporins.

Despite the discovery of this powerful new antibiotic, we still believe that we should continue our efforts in this field and that a sophisticated research programme would lead to new, completely different and even better drugs. In this sense, I hope that we will have today interesting speeches and fruitful discussions among the scientists present here.

I pass the word now on to the two Chairmen. The first session is moderated by Dr. Reden, the second one is chaired by Dr. Divekar.

I wish this Symposium great success.

NEW WAYS TO NEW ANTIBIOTICS

by

Prof. H. Zaehner

Professor H. Zaehner was born in 1929 in Zurich, Switzerland.

He studied Agricultural Science at E.T.H., Zurich where he acquired his Ph.D. in 1954 with a thesis in the field of phytopathology.

From 1954 to 1958, he worked at the Institute of Botany at E.T.H. in Prof. Ettlinger's group, whose department he subsequently took over in 1958. By then his main task was to search for new antibiotics in close co-operation with Ciba AG., Basel and the Organic Chemistry Laboratory of the E.T.H.

In 1960, Prof. Zaehner got his habilitation. Since 1964, he is Professor of Microbiology at the Tubingen University, Germany.

First two provocative questions :

1. Do we need new antibiotics?
2. Do we need new ways to find antibiotics?

Table 1 shows a list of the antibiotics now on the market. This list is very long, but the list of pharmaceutical preparations containing antibiotics would be much longer. These lists predispose us to say, very quickly and emotionally, that we do not need new antibiotics. A more close analysis of the situation, which cannot be covered in this lecture, gives another picture. In table 2 you find a compilation of the possible fields of antibiotic applications. Only one field seems to be completely covered by available antibiotics, namely, the infections of man with Gram positive bacteria. All other fields are virtually uncovered. The answer to the first question must be, we do need new antibiotics, even though the first reaction was negative.

Do we need new ways to find these necessary new antibiotics? A lot of antibiotics were found by the classical methods, which were established by Florey, Waksman and other authorities 40 or more years ago. From 1941 to 1956 a lot of useful antibiotics were found and this justifies the question, do we need new ways?

Already in 1958 Woodruff and others admitted that they had isolated only one new compound from 1000 screened strains.

Table 1

Year of initial report	Now used in clinical medicine		Now used in agriculture
Before 1945	Actinomycin D	Penicillin G	
	Bacitracin	Penicillin V	
	Gramicidin D	Streptomycin	
	Griseofulvin	Tyrothricin	
1946—1950	Chloramphenicol	Neomycin	Antimycin A
	Chlortetracycline	Oxytetracycline	Cycloheximide
	Colistin	Polymyxin B	Nisin
	Fumagillin		
1951—1955	Actinomycin C	Nystatin	Staphylomycins
	Amphomycin	Oleandomycin	
	Candicidin B	Sarkomycin	
	Cycloserine	Spiramycin	
	Erythromycin	Staphylomycins	
	Gramicidin J	Tetracycline	
	Leucomycin	Trichomycin	
	Lucensomycin	Viomycin	
1956—1960	Aminosidin	Novobiocin	Blasticidin S
	Amphotericin B	Paromomycin	Cellocidin
	Chromomycin A3	Pentamycin	Hygromycin B
	Demethylchlortetracycline	Pimaricin	Mikamycin
		Pristinamycins	Stendomycin
	Hamycin	Rifamycin B	Thiortrepton
	Kanamycin	Ristocetin	Tylosin
	Mithramycin	Spectinomycin	
	Mitomycin	Vancomycin	
		Variotin	
1961—1965	Azalomycin	Gentamicin	Aureofungin
	Bleomycin	Lincomycin	Destomycin
	Capreomycin	Pyrrolnitrin	Kasugamycin
	Cephalosporin	Siccanin	Moenomycin
	Daunomycin		Polyoxin
	Fusidic Acid		Siomycin
1966—1970	Adriamycin	Ribostamycin	Monensin
	Enduracidin	Tobramycin	Validamycin
	Josamycin		
1971—1975	Maredamycin	Lividomycin	Lasalocid
	Bicyclomycin	Sisomicin	Macarbomycin
			Mocimycin
			Salinomycin
			Thiopeptin

Table 2

Possible fields of antibiotic application

Occupied fields	Partially occupied fields	Open fields
Medical fields		
Gram positive bacteria	Gram negative bacteria dermatophytes	systemic mycosis virus infections cancer parasites
Non-medical fields		
	fungal infection of plants	bacterial and viral infection of plants insects, mites and nematodes in plant pathology animal nutrition food conservation technical application

This frustrating result confirms our experience.

In our screening, in 1958, only one of 50 investigated antibiotics had proved to be new. Since 1958 the situation has worsened. The newly found compounds are not new types of antibiotics but, in most cases, only new members of old, well investigated families of antibiotics, for example, a macrolide, a new polyene, an anthracycline, etc.

If the search for new antibiotics should not be a work of Sisyphus we have to look for new ways. These can be found in four different directions:

1. Through antibiotic producing micro-organisms.
2. Through modifications of established antibiotics.
3. Through novel tests methods of detection.
4. By alternative ways to new compounds.

1. Possible ways to new antibiotics through antibiotic producing micro-organisms

The distribution of the known antibiotics is extremely unequal. More than 50% of all antibiotics are produced by only one genera, the streptomycetes. No antibiotics have been found in yeasts. Waksman has opened a new field by the introduction of the actinomycetes into antibiotic research. One factor in this success was the improvement of culture techniques in this group of micro-organisms, the other major advance being his excellent work in the systematic classification of these micro-organisms. If a new group of micro-organisms should be introduced in the field of antibiotic research both requirements must be fulfilled, not necessarily by the same people but with the same aim. The progress of the cultivation of micro-organisms, which for so long was so difficult to achieve, is rapidly increasing and it should be possible to introduce some new groups in our field of research. But we have to take care that the knowledge about the systematic classification of these organisms achieves the same level, so that a chemotaxonomy can be established. Possible candidates for introduction in this field would seem to be some basidiomycetes, myxobacteria, myxomycetes, some protozoa and so on.

The introduction of other microorganisms as sources of antibiotics is not the only way to enlarge the possibilities on the side of microorganisms. If we compare the methods of cultivation used in the laboratories, we realise that in most cases all groups use very similar or identical methods. The screening is done very schematically in a way that ensures formation of antibiotics in a fixed manner, independent of the culture conditions. By a variation of the culture conditions such as pH_2, pCO_2, temperature, osmophilic conditions, composition of media, addition of inhibitors or feeding in of precursors the spectrum of formed antibiotics can be enlarged dramatically. In certain cases the very efficient regulation of the intermediary metabolism hides the ability to form secondary metabolities. *Candida lipolytica*, for example, cultivated under normal conditions forms no antibiotic or, more accurately, no easily detectable amounts of antibiotic. If we feed in tryptophan and deregulate the cell's own regulation of the tryptophan-biosynthesis, we can isolate the antibiotic Tryptanthrin.

The antibiotic was recently identified as an alkaloid from two different plants. The culture conditions are not fixed, they can be changed and thus, the pool of antibiotics is enlarged. The genetic base of micro-organisms is also not of a fixed size, and the genetic base can again be changed. Modern genetics of micro-organisms enbrace many methods for modifications of the genetic base such as mutations in primary and secondary metabolism, crossing of strains, transduction by phages or plasmids, and fusion of protoplasts. All these are available even if we do not want to use the methods of genetic engineering. We have only preliminary experience with these methods in the field of antibiotic screening. We do not know if the spectrum of formed antibiotics can be enlarged easily by these means and I do not participate in the immense enthusiasm of some of my colleagues even though I think the new methods can be very helpful in the search for new antibiotics.

2. New antibiotics by modifications of established structures

The first generation of antibiotics was marketed in their native form, the form in which the compounds were produced by the micro-organisms. Today, most antibiotics come on the market in a modified form: for example, the semi-synthetic penicillins, semi-synthetic cephalosporins, derivatives of Kanamycin, etc. A chemical modification can change a lot of properties of the native compounds: a look at the semisynthetic penicillins illustrates the possibilities. Chemical modification is not the only way to achieve a modified new compound. A combination of chemical, microbial and genetic methods can lead also to new compounds. I will give some examples:

a) The feeding in of modified precursors to wild strains or to special mutants leads in some case to new compounds. The formation of Penicillin V by feeding in of phenoxy acetic acid is an old example, the new aminoglycosides formed after feeding in of modified deoxystreptamines to deoxystreptamine minus mutants are new ones.

b) The modern methods of separation such as the preparatory HPLC allows the investigation of very complicated mixtures. In the earlier stage of antibiotic research people had to look for strains with a small number of components; today, it is possible to investigate strains with very high number of components. This leads at an early stage to a lot of information about the possible variation of the molecules in nature and to the first discovery of the relation between structures and activity. Some of the biological variants have structures which cannot be prepared easily by chemical methods. But a major problem for those using this method cannot be overlooked. If one of the minor components is of major interest it will be necessary to look for mutants or fermentation conditions in which this minor component is now a major component. That may be an insoluble task.

c) Micro-organisms are able to transform a lot of different substances, including antibiotics. Even though the microbial transformation of steroids has an old tradition in the pharmaceutical industry, this method is not used for the preparation of new antibiotics. The microbial transformation of antibiotics is well investigated in relation to antibiotic resistance but not so far as a possible way to find new compounds.

The methods I have just described lead to modified new members of an antibiotic family, but they do not lead to new groups of antibiotics.

A further consideration should be mentioned at this point. Cephalosporin C itself is not a useful antibiotic but it could be modified to form very good derivatives. The derivatisation of Cephalosporin C was started because of the great success with the penicillins. Without the knowledge gained from the penicillins, Cephalosporin C would, perhaps, have been neither investigated nor modified. Antibiotic literature refers to some other antibiotics which could be useful if they were modified appropriately. It seems time to look at some old antibiotics and to think about a modification of these molecules. A further positive example is Pleuromutilin: a derivative of it was recently made commercially available for treatment of animals suffering from mycoplasma infections. But how should we determine the essential indication for the selection of the right antibiotic for such a work? Indications for selection could be the toxicity, the mode of action, the intensity of action and some chemical aspects.

3. New antibiotics by the use of novel detection methods

Two factors determine what can be found in a screening of antibiotics, the micro-organisms and the method of recognition. This statement is trivial but, nevertheless, the importance of recognition methods was overlooked over a long period. The new β-lactams are a very good example of the importance of the recognition methods. In 1941, the first chemical report on Penicillin was published and in 1956 came Cephalosporin C. (Table 3). But Cephalosporin C was, in fact, a minor product which would not have been detected at that time in a conventional screening programme (E.P. Abraham, 1979).

From 1941 to 1970 a lot of people were looking for new antibiotics and we can be sure that they would have published the discovery if they had found a new β-lactam. No new β-lactam was reported from 1956 to 1971 and it seemed that all β-lactams in existence had been found. The scene changed completely in 1972. What has happened since 1972? Since that year, new methods of recognition were introduced into antibiotic research. The use of β-lactam supersensitive strains, the search for inhibitors of β-lactamase and also for sulphur containing compounds led to a series of new compounds. The pool of β-lactams is, by no means, exhausted. I do not know if these new β-lactams such as Thienamycin, Clavulanic acid, Nocardicins, etc. can be modified with the same success as the penicillins and cephalosporins, but I think that the new β-lactams illustrate convincingly the importance of recognition methods. It seems to be worthwhile to re-evaluate our methods for recognition of microbial metabolites.

Waksman and other authorities on antibiotic research have proposed very simple methods for antibiotic detection. The classical methods use micro-organisms for the primary test which are as closely related as possible to the later application. This correlation to the later application was, and is still, an essential factor in the choice of the primary test in most laboratories. Today, this correlation is still required by many people, such as Frommer et al. and others, but a look of the new β-lactams shows that these were found by very special tests; for example, tests for sulphur-containing compounds or tests with β-lactam supersensitive strains and nobody has thought of using these against an infection with β-lactam supersensitive bacteria. The tests used for the recognition of the new β-lactams are very sophisticated, but they are useful only for the detection and have no direct correlation to the planned later application. We should dispense with this direct correlations as quickly as possible. Here are some reasons for this opinion:

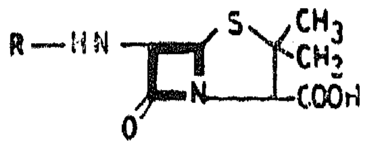
1. The need of a correlation is restricting us very greatly because of the choice of the method for recognition.

2. The correlation is not as accurate as we thought. Even in the best case, the infection of man by Gram positive bacteria and the use of these bacteria for a primary test is recorded as having a correlation coefficient smaller than 0.1.

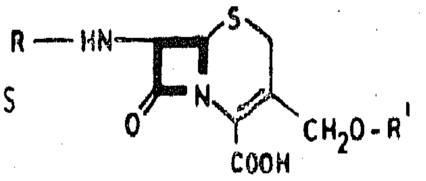
Table 3

β-LACTAMS

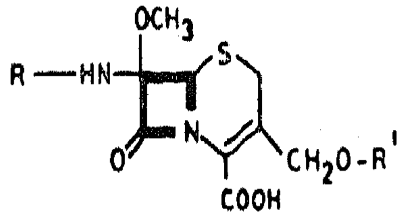
PENICILLINS
(1941)



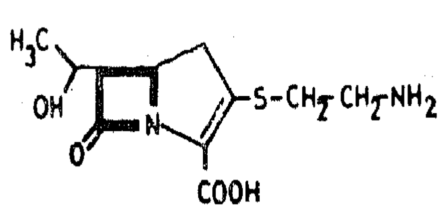
CEPHALOSPORINS
(1956)

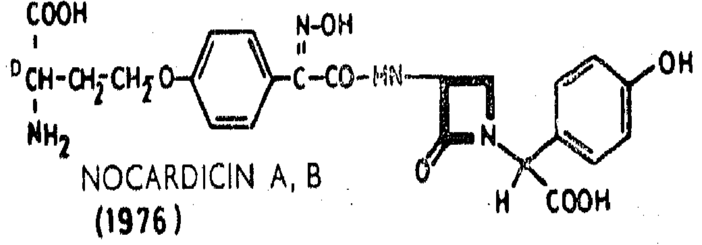


CEPHAMYCINS
(1972)



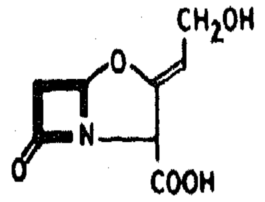
THIENAMYCIN
(1975)



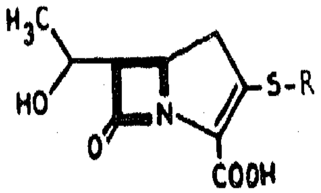


NOCARDICIN A, B
(1976)

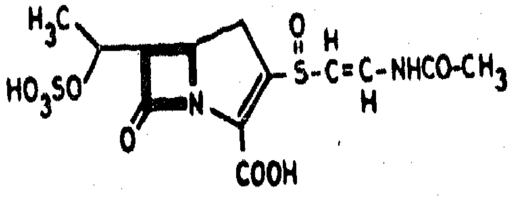
CLAVULANIC ACID
(1976)



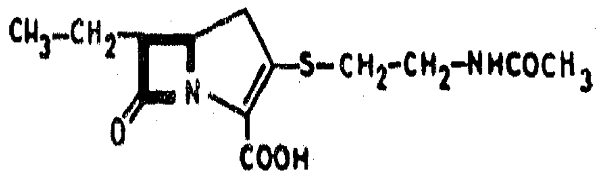
OLIVANIC ACID
(1977)



MC 696-SY2-A=MM4550
(1977)



PS-5
(1978)



3. We have a large number of fields open for an antibiotic application and we have in each case a correlation to only one field. Most of the teams doing the screening would be looking for antibiotics in differing fields.

4. For many very important applications no correlation exists between the primary test and the desired application.

5. New metabolites are wanted not only for chemotherapy of infections but also for numerous other applications in medicine, for example, allergy, blood pressure, cancer.

6. If we dispense with this correlation we can choose a test which reduces our problems of identification of the activities discovered.

7. Fewer tests are required when we feed into a screening only biologically active, virtually pure compounds for the tests related to the later application. This smaller number of tests can be made much more carefully than in a classical screening. A test done with pure compounds gives more information about activity than a test made with an unknown compound in an unknown concentration and in combination with an unknown number of other unknown factors.

8. Some of the more sophisticated tests for pharmacologically active compounds can only be done with more or less pure compounds and not with culture filtrates or crude extracts.

The changes in screening by the methods I have sketched lead to a change in focal points. Focal points in a classical screening are the isolation of a great number of different micro-organisms and the identification of the found activities. In the alternative method of screening, the focal point will be the isolation of compounds and their analysis. The limitating factor will be the preparation of the first 100 mg of pure compound and, afterwards, the preparation of greater amounts for a larger evaluation.

Table 4 summarizes some possible test methods for detecting and isolating secondary metabolites, without a correlation to a later application. I am able to give some illustrations from our laboratory.

Table 4

Some methods for recognition of secondary metabolites

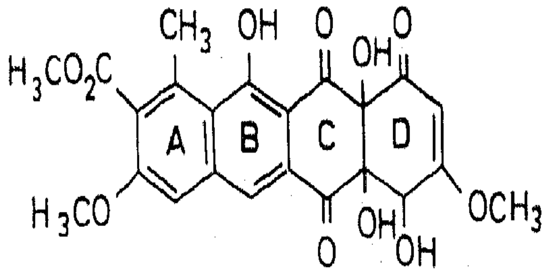
Antimicrobial tests
  "classic tests" with bacteria or fungi related to pathogenic strains
  with supersensitive strains
  of substances with a small action spectrum
  in combination with EDTA or detergents
  with cultures of tumour cells
  in combination with antibiotic and antibiotic resistant strains
Tests for biological activities other than growth inhibition
  irregular growth of fungal mycelium
  inhibition of spore formation
  inhibition of chemotaxis or phototaxis
Cell free test systems
  protein biosynthesis
  enzyme inhibitors for protease, chitinase, sialidase, tyrosine-hydroxylase a.s.o.
  membrane permeability
Chemical test systems
  thin-layer chromatography with specific reagents for certain chemical groups
  high-pressure liquid chromatography, with polarographic or photometric determination
  incorporation of radioactive isotopes such as ^{32}P, ^{36}Cl or ^{35}S

Test for metabolites with a small action spectrum. The test is based on the following concept: The well-known antibiotics inhibit Gram positive and partially Gram negative bacteria. With a test containing *Bacillus subtilis* and perhaps *Streptomyces viridochromogenes* or *Clostridium pasteurianum* we can select antibiotics without activity against *Bacillus subtilis* but with high activity against one of the other strains. These antibiotics with a small spectrum of activity are, probably, new because in the old tests with the bacteria normally used these antibiotics did not appear. We have been doing this test for more than 10 years and we have thus isolated a series of antibiotics. Our latest findings are as follows :

The tetracenomycins

The rare strain *Streptomyces glaucescens*, which has been relatively well investigated genetically, produces, besides the known oxy-streptomycin, a family of new antibiotics, the tetracenomycins (Fig. 1). These yellow substances show only a small spectrum of activity. The investigation of their chemical structures leads to the following results:

AMO - 290



Tetracenomycin C

Fig. 1

The tetracenomycins are, from the point of view of their chemistry, classified between the tetracyclines and the anthracyclinones. Both families are very important antibiotics, the former for the chemotherapy of infections with Gram positive and Gram negative bacteria and the latter in the form of their glycosides for tumour chemotherapy. A preliminary study of the mode of action shows that the tetracenomycins probably act in a way which is quite similar to the anthracyclines and we are impatiently awaiting the results of the tests of tumour chemotherapy. We hope we have found in the tetracenomycins a group of compounds which merit more extensive chemical research in relation to tumour chemotherapy.

However, new ways do not lead automatically to new compounds: sometimes they lead to old ones. We have for example re-found Cladosporin. Cladosporin was reputed to be a mild antifungal antibiotic with a minimal inhibition concentration of 40 μg/ml. In our tests Cladosporin is active against a small range of bacteria at 0.02 μg/ml. The identification necessitated a lot of work because the antibiotic was not recognised to be Cladosporin. Only after a complete elucidation of the structure by mass-spectroscopy and NMR we realized that the substance was in fact Cladosporin.

b) Tests with criteria other than growth inhibition. Every easily measurable biological reaction can be chosen for this type of test. We have some experience with tests on a typical growth of mycelium of fungi and with fairly selective inhibition of formation of sexual spores.

Ansatrienin (Fig. 2)

Using *Botrytis cinerea* as the test organism the formation of a typical hyphae can be quantitatively registered. We used this test more than 15 years ago. Some new antibiotics were found with this test such as Boromycin, Venturicidin B, Scopamycin, Scopathricin. The newest example is a triene, but the triene part is incorporated in an ansa ring. The antibiotic shows some similarities to Geldanamycin and also to the Maytansins.

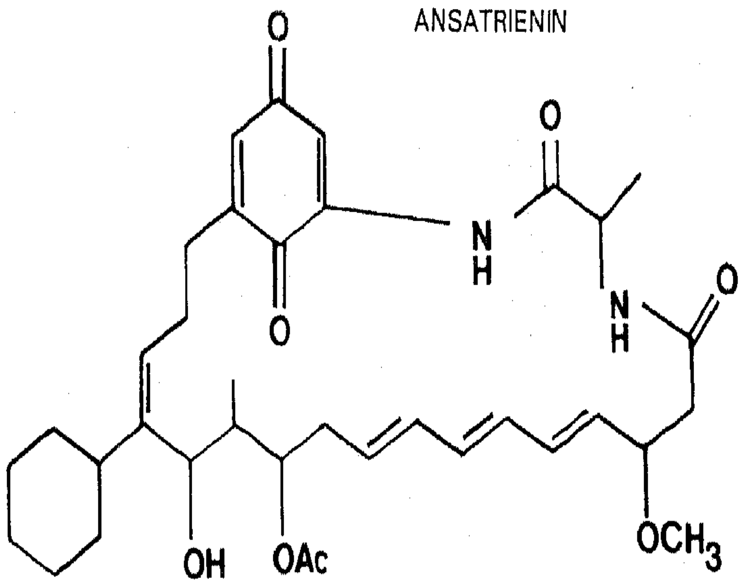


Fig. 2

The Nikkomycins

By using a strain of *Mucor mihei* with dark brown zygosporos an inhibition of zygospore formation can be recorded together with an inhibition of mycelium growth in the same test. The Nikkomycins were found by this test. *Streptomyces tendea* produces a large number of similar nucleoside antibiotics of which not all components have yet been isolated. A combination of ion exchange resins, Sephadex LH 20 and a semipreparative HPLC leads to the isolation of 3 major components X,Z,I and some minor components.

The chemistry of the Nikkomycins, investigated by Prof. Hagenmaier's and Prof. Konig's teams, is summarized in Figure 3.

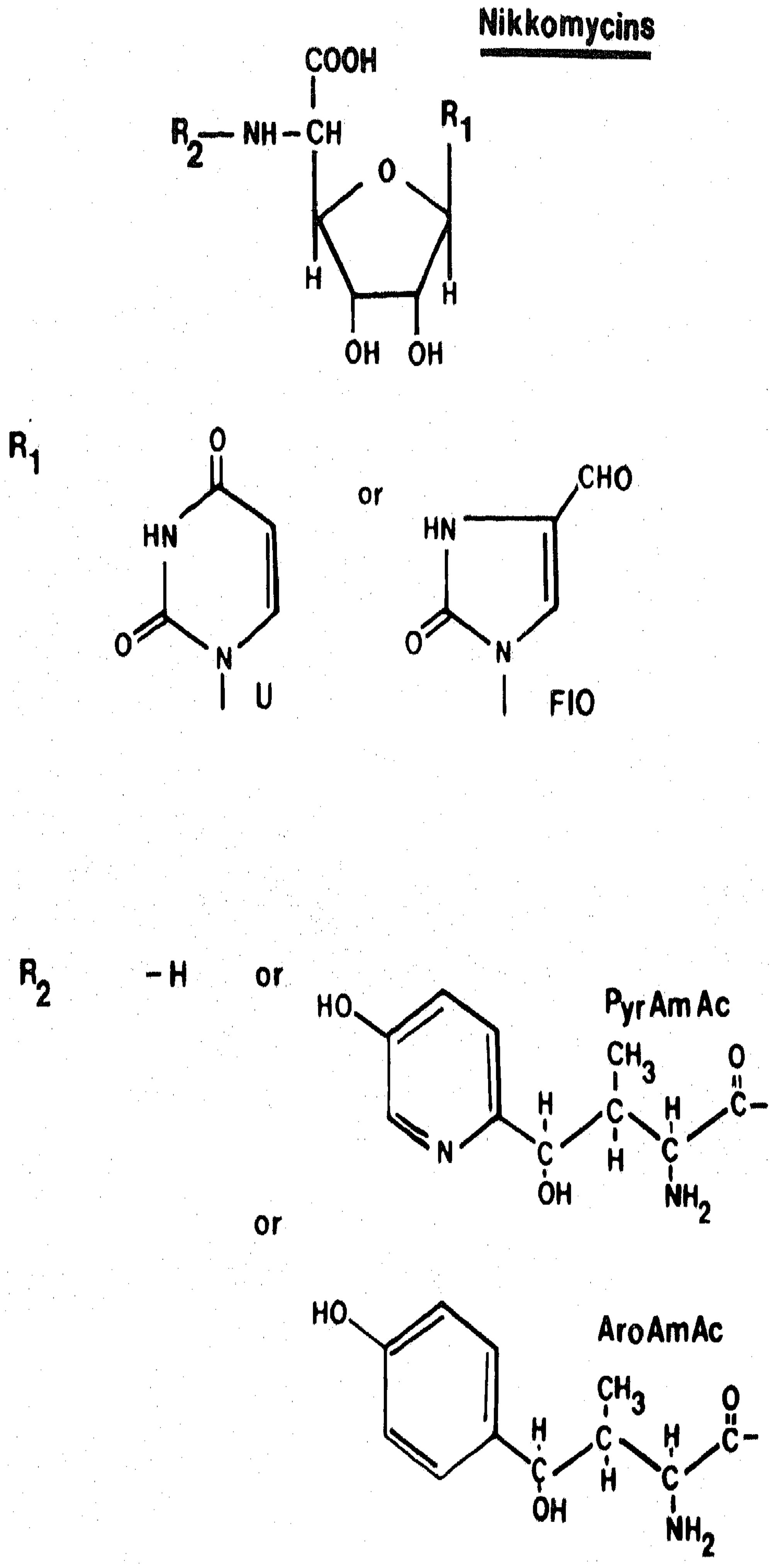


Fig. 3

An amino uronic acid seems to be the unique element which is the same in all investigated Nikkomycins. The substituent R_1 can be Uracil and an isomer of Uracil though this latter has yet to be identified and also the 4-formyl-imidazolon-2-on. R_2 can be H or one of the new rare amino acids.

The Nikkomycins are very active antibiotics, active against fungi, if the fungi have chitin as a major component of their cell wall. The antifungal activity is higher than the activity of Polyoxins by a factor of 10-300 and the Polyoxins have been put on the market. The Nikkomycins also show a good activity against insects and mites. We hope we have found in the Nikkomycins a group of useful compounds for plant protection. The mode of action investigated by Dahn and Coworker in 1976 and Brillinger 1979 showed that it is an inhibitor of the last enzyme in chitin biosynthesis, the chitinsynthetase. Higher plants and mamalian cells do not contain chitin and for this reason a low toxicity could be expected. In fact, preliminary experiments showed no toxicity in rats and mice and only a very low degree for plants.

If we dispense with the correlation between the primary tests and the later application then we do not need to include a biological test for the recognition of new metabolites.

We can achieve the same aim using chemical methods. Thin-layer chromatography on silica gel with different solvents and in combination with a set of different chemical reagents for the detection of spots is a relatively simple method which can be used for the screening of crude extracts from microorganisms. We, so far, have only preliminary results from this method, but these results are encouraging us to continue. In a preliminary screening we have isolated 5 compounds from less than 50 strains of streptomycetes. One of these five compounds was a known antibiotic, overlooked in the usual screening, another was the amide of anthranilic acid and three compounds which seem to be new. For two of these three compounds we have the structure: Ketalin (Fig. 4) is a relatively small molecule, whose structure has been established by Prof. Konig. This compound acts as an ionophore in the test with liposomes but the compound does not demonstrate any antimicrobial activity and the compound leads to a stimulation of the lymphocytes which corresponds with its effect as an ionophore, mentioned earlier — Ferrithiocin (Fig 5) is a sulphur containing compound produced by a strain which forms Rubromycins. The sulphur compound shows antibacterial activity which is due to the strong chelating effect of the iron. The antimicrobial action is comparable to those of nocardamine and deferri-tri-acetyl-fusigen. We have still to discover whether this compound possesses other biological activity or if this substance can be used for the therapy of iron overload disease. The structure of the third compound is not known. The compound demonstrates an ionophoric activity on liposomes without any antimicrobial action.

Three new compounds isolated from less than 50 strains, and every isolated compound with a biological activity, is a very satisfactory result for a screening, even

The structure of ketalin

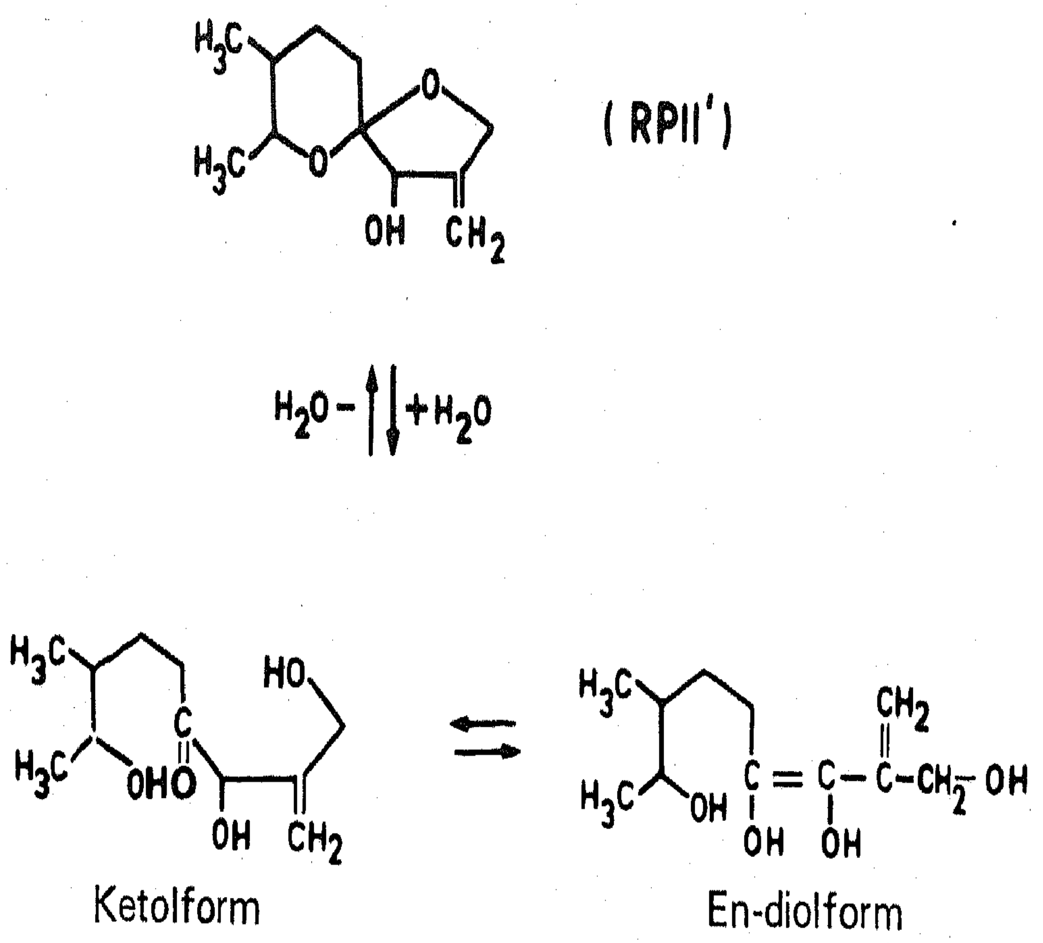
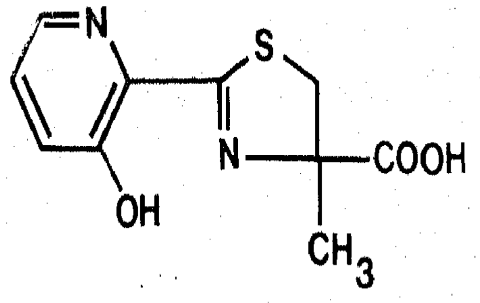


Fig. 4



DESFERRI-FERRITHIOCIN

Fig. 5

though we have chosen in these first experiments a very small number of the detected spots from the thin-layer chromatography. It would have been possible to isolate many more compounds from the same screening then we have yet done. The ratio of 3 new compounds out of 5 isolated compounds is encouraging us to a more extensive use of this method. One advantage of the method is the rapidity with which the test can be done and this allows a better control of the fermentation and a much easier isolation than the normal biological test, which takes 16 hours and more.

I will risk making the statement that more than 10-20 new compounds from 100 different actinomycetes strains could be found by this method. And I will take a further risk by saying that every isolated microbial compound possesses a biological activity. The only question is to detect where the supposed activity is to be found. If we are interested in biological activities on a wider range and for different applications, it seems to be much easier to isolate new compounds by this technique combining both chemical and microbiological methods and then carefully evaluating in many different fields, rather than by a screening using different primary tests with a more or less unknown correlation to the later applications.

The choice of an optimal reaction for the detection of spots is one critical point, the selection of the "best" spot for the isolation is another. The progress of chemical analysis would allow either now or in the near future the virtually complete investigation of all microbial metabolites formed by one strain. For lovers of completeness it must be a fascinating possibility to be able to isolate all compounds by using an HPLC, with a still not available C-detector, in combination with mass-spectroscopy. At first sight it would seem to be only a hobby for fanatical advocates of HPLC but, in fact, it will be the only feasible way. If the results from the HPLC analysis could be transformed to a preparative HPLC, the very high expense for the primary screening could partially be saved by a very rapid preparation of the first 500 mg of the new compound needed for a first evaluation and an extended chemical characterisation. There is one problem which should not be overlooked, the need of a very high degree of standardisation of the culture of micro-organisms which are fed into such a screening.

4. Alternative ways to new compounds

The search for new antibiotics with old or new test methods, old or new microorganisms resembles the hunt for the needle in a hay-stack. However, one may be the happy man who strikes oil in his back garden. To use a Swiss metaphor, one may be the blind chicken who finds a corn.

It is, I hope, understandable that we should be looking for another approach. Some of the molecular biologists had hoped that, in the near future, they would be able to synthesize new useful compounds according to their needs. But it was only a dream.

And I am going to present to you another dream. We are always surprised how large an antibiotic can be and how low the minimal inhibition concentration of a good antibiotic is. The idea that antibiotics can not only enter the cell in passive diffusion but that they should be taken up by an active transport or facilitated diffusion is too revolutionary to be accepted at first hearing. The idea that bacterial cells are going to take up actively a compound which will kill the cells is not consistent with our view of antibiotics. But we have a lot of observations that some antibiotics can be taken up by active transport. (Table 5)

Table 5

Antibiotics	"Misused" Transport System
sideromycins:	sideramin transport system
albomycin (grisein)	
ferrimycin	
A 22765	
succinimycin	
tripeptides:	oligopeptide transport system
phosphinothricin-ala-ala,	
L-(N^5-phosphono) methionine-S-sulfoximinyl-ala-ala	
plumbemycin B	
dipeptides:	dipeptide transport system
bacilysin	
D-cycloserin	D-alanine transport system
phosphonomycin	transport system for glycerophosphat + system for glucose-6-phosphat
showdomycin	cytidine transport system
streptozotocin	PEP-sugar-phosphotransferase system, part specific for N-acetylglucosamine
nojirimycin	PEP-sugar-phosphotransferase system, part specific for glucose
streptomycin	polyamine transport system

The bacterial cell does not possess special transport systems for antibiotics. The antibiotics are appropriating some of the existing systems for substrate transport. In some cases it was possible to identify the substrate transport system which is taken over by certain groups of antibiotics. When we know enough about the transport systems stolen by antibiotics, it should be possible to design new drugs, or to modify a known inhibitor in such a way that the inhibitor can enter a larger spectrum of bacteria or accumulate in their cells, which leads to a higher activity. A comparison of the natural substrate and the antibiotics misusing the transport system of this substrate gives us some information about the part of the molecules which cannot be modified without losing their capacity for being transported, and the other part of the molecule which can be modified in many ways if some limits of its size and its functional groups are considered.

A new inhibitor designed by this hypothesis would have two parts: A vehicle and a war head.

The pertinent questions are: What can be a useful vehicle and what can be an optimal war head? The simplest vehicle seems to be some amino acid if the war heads are also amino acids (Fig. 6). The dipeptide compounds formed would be transported by the dipeptide — and tripeptide systems and split in the cell by its own peptidase into the normal amino acid, which was only one part of the vehicle and the unusual amino acid which is the active war head. The good results of this group (Ringrose *et al.*) show that the idea of combining a war head with a vehicle is very likely more than only a dream (Fig. 7).

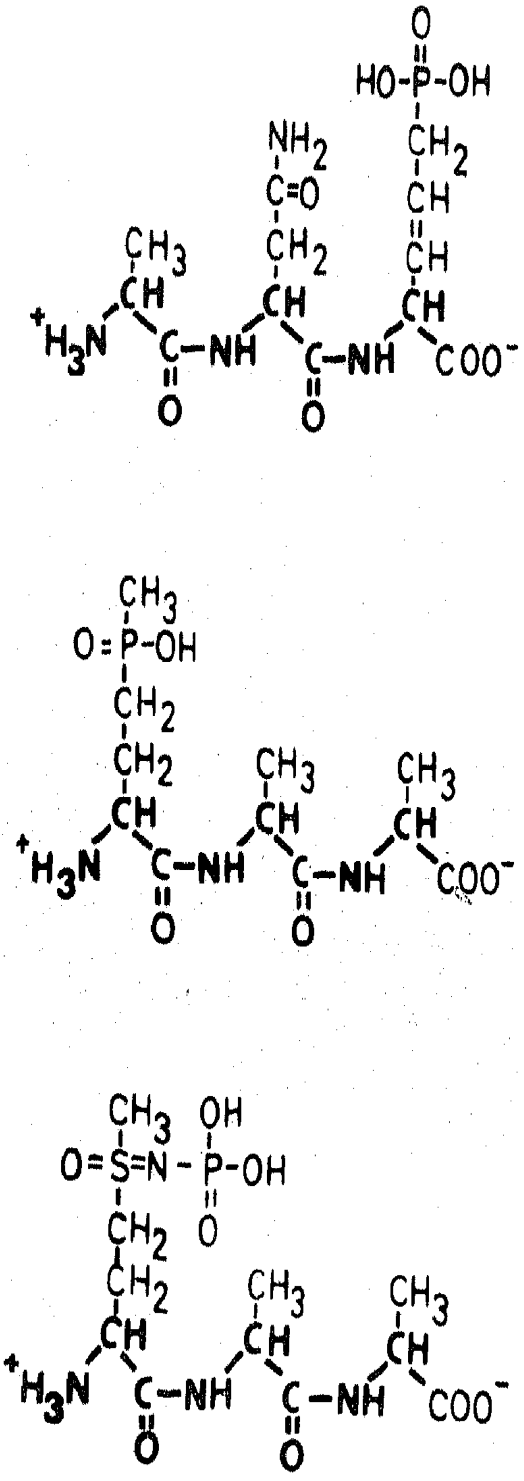
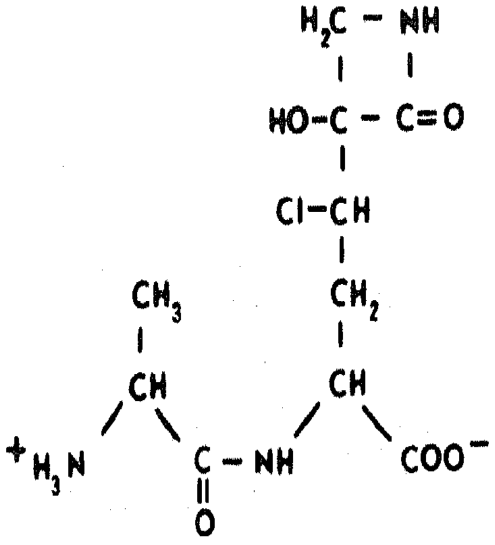
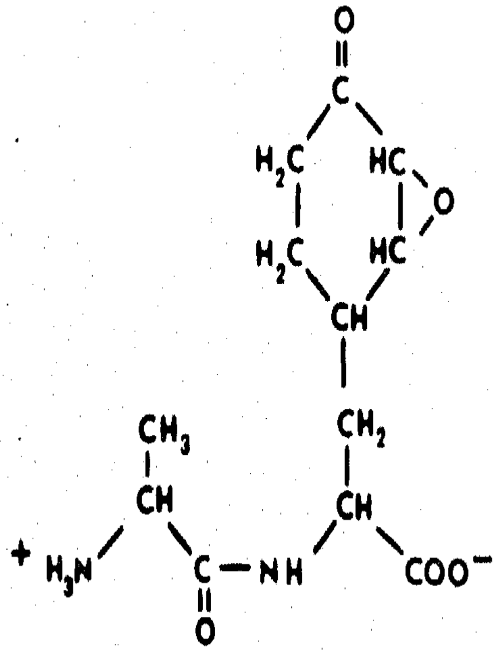


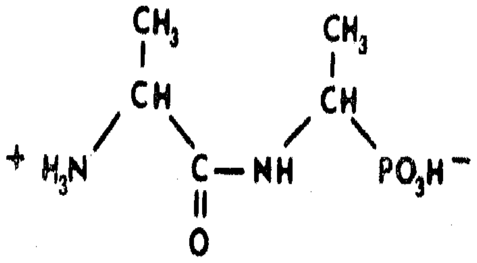
Fig. 6



(S)-Alanyl-3 [a-(S)-chlor-3-(S)-hydroxy--2-keto-3-azetidinylmethyl]-(S)-alanin



Bacilysin



Alaphosphin

Fig. 7

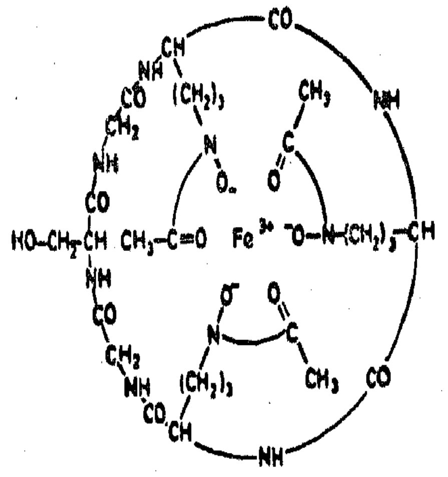
Sideramines, natural and semisynthetic sideromycins

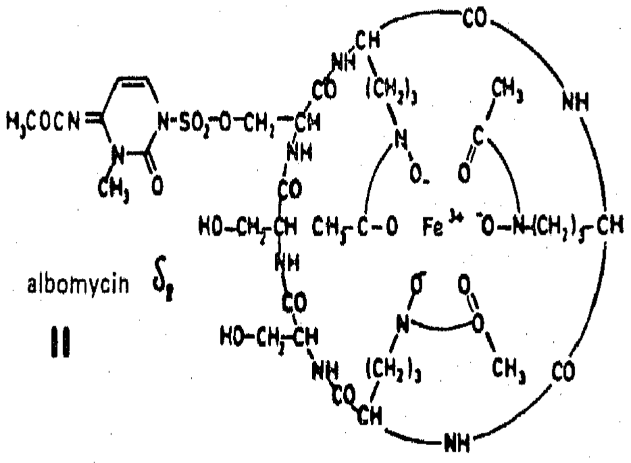
A. ferricrocin series

Sideramine

ferricrocin

I





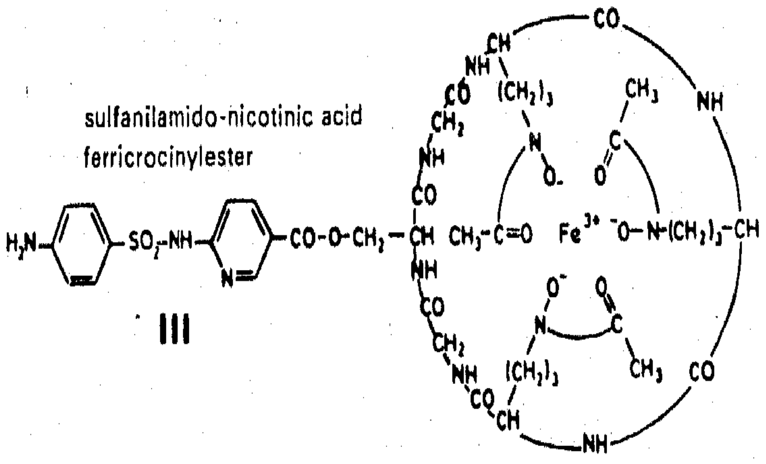
Natural sideromycin

albomycin δ_2

II

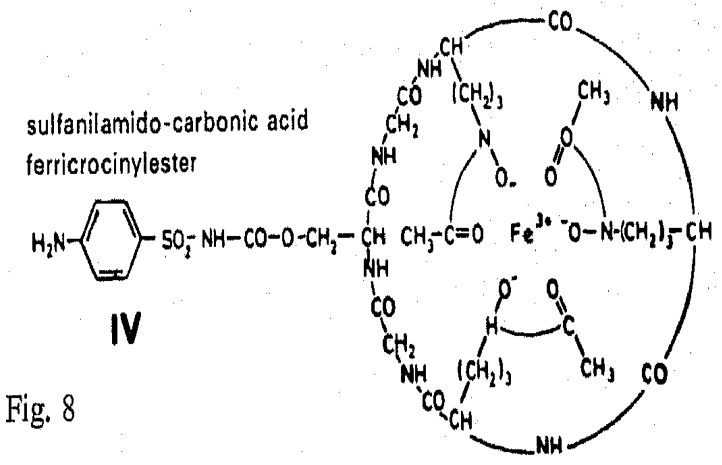
Semisynthetic sideromycin

sulfanilamido-nicotinic acid ferricrocinylester



III

sulfanilamido-carbonic acid ferricrocinylester



IV

Fig. 8

Another much more complicated vehicle could be the Sideramines. Koller-Schierlein and Nageli have synthetized some semi-synthetic Sideromycins with a sulphonamid group as a war head. When the war head was linked by a spacer the compounds were active. These new Sideromycins were transported by the Sideramine transport system and they can be antagonised on this transport system by Sideramines (Fig. 8). At the target the new Sideromycins act as sulphonamides and this mode of action can be antagonised too, in this case by para-aminobenzoic acid.

Holtje has demonstrated that the aminoglycoside antibiotics induce the polyamine transport system and are taken up to the second phase of uptake by this system. It seems possible that polyamines could be good vehicles too, in this case probably for fairly specific use with Gram negative bacteria because Gram positive bacteria do not possess an active polyamine transport. It has not yet been discovered whether this vehicle can be used to transport war heads.

The search for other vehicles as well for other war heads has just started and a lot of surprising results may be expected.

At the end of this lecture, which was restricted to the practical use of antibiotics I should like to mention some more theoretical problems which are nevertheless highly relevant to our view of antibiotics. 50 years after the discovery of the penicillins we have still not discovered the function of this compound for the producing cell. And the same is the case with most of the other antibiotics. We only know that the antibiotics suggested earlier as being weapons in the fight against invaders can no longer be accepted.

Antibiotic research cannot be done by one person alone and it should be made clear that the results here reported are the achievement of many people. The antibiotics mentioned here were the result of a close collaboration between colleagues from the Institute of Biology II and the Institute of Organic Chemistry of the University of Tubingen and the Institutes of Organic Chemistry from the Universities of Gottingen, Hamburg and the ETH Zurich.

RECENT ADVANCES IN SEMI-SYNTHETIC β-LACTAM ANTIBIOTICS

by

Dr. J. Coombes

Dr. J. D. Coombes obtained his B. SC. degree in Physical Chemistry in 1953 and a Doctorate in Organic Chemistry in 1956, both from the University of Bristol.

He was appointed Research Fellow in Chemistry at Harvard, U.S.A. in 1956. Dr. Coombes returned to the United Kingdom three years later to take up an appointment as Lecturer in Biophysics at King's College, London University.

Joining Pfizer Limited in 1962, he eventually became Director of Chemotherapy and Biological Research.

Dr. Coombes joined Hoechst U.K. in 1973, as Head of the new Milton Keynes Research Laboratories.

In my presentation today I want to outline the work that has gone on in recent years on the modification of naturally occurring β-lactam antibiotics to produce semi-synthetic derivatives with advantages over the naturally occurring compounds. I shall bring my story up to the present and indicate where current efforts are directed, which may be expected to yield new and superior agents in the years to come.

It is obviously not possible for me to review the whole field of β-lactam antibiotics, and I shall instead try to highlight the successful chemical strategies adopted to try and achieve biological goals. I shall also present the story with the benefit of hindsight so that things may appear more logical and rational than was the true situation at the time.

The basic ring structure of the naturally occurring β-lactam antibiotics contains the following features:

1. Both substances contain the 4 membered azetidinone ("β-lactam") ring.
2. Both substances have an amine group attached to the β-lactam which is condensed with an acid to form an amide side chain. In both substances the stereo-chemistry of this side chain is the same (cis).
3. Both substances have S and COOH groups in the same juxtaposition to the β-lactam ring.

4. The heterocyclic ring in the penicillins is saturated, that in the cephalosporins unsaturated.

The earlier modifications of the penicillins and cephalosporins were, of course, the introduction of new side chains on to the basic 6APA or Cephalosporin C starting materials available from fermentation. I will return to this area later but, first, I should like to discuss nuclear modifications of penicillins and cephalosporins, by replacing the S with other hetero atoms.

Nuclear Modified Cephalosporins

The nomenclature of these substituted derivatives is shown in Figure 1.

NOMENCLATURE

REPLACEMENT OF SULPHUR IN NUCLEUS OF PENICILLINS AND CEPHALOSPORINS BY

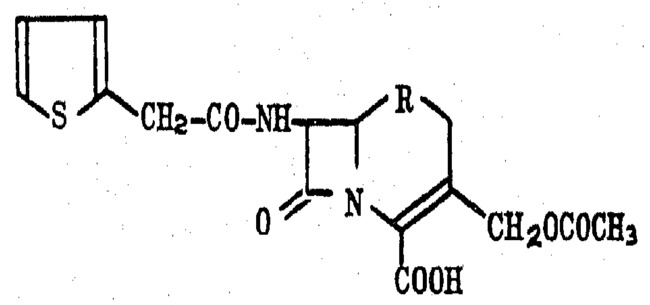
CARBON → 1-CARBA -
OXYGEN → 1-OXA -
NITROGEN → 1-AZA -

Fig. 1

The first nuclear modified cephalosporins were made by Christensen of Merck Sharp and Dohme. Tables 1 and 2 show the activity of some of these substituted cephalosporins. It should be pointed out that the synthetic route was non stereospecific so that both enantiomers were present in the modified materials. Both tables show that 1-oxa and 1-carba analogues of cephalothin and cephamandole are active.

Table 1

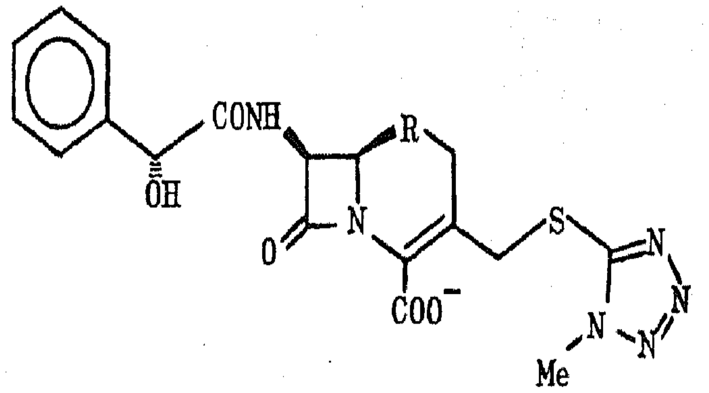
ACTIVITY (MIC) OF CEPHALOTHIN ANALOGUES



ORGANISM	R		
	S(CEPHALOTHIN)	O	CH_2
S.AUREUS 2865	< 0.39	< 0.39	< 0.39
S.PYOGENES 3124	< 0.39	< 0.39	< 0.39
KLEBSIELLA sp 2882	3.12	6.25	0.78
E. COLI 2884	6.25	6.25	< 0.39
SHIGELLA sp 2880	3.12	6.25	-

Table 2

ACTIVITY (MIC) OF CEPHAMANDOLE ANALOGUES

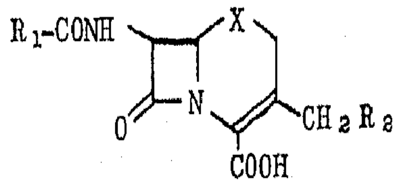


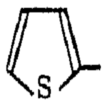
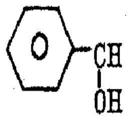
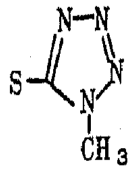
ORGANISM	R		
	S(CEPHAMANDOLE)	0	CH$_2$
S.AUREUS 2855	< 0.39	1.56	1.56
S.PYOGENES 3124	< 0.39	< 0.39	3.12
KLEBSIELLA 2882	1.56	6.25	1.56
E.COLI 2884	< 0.39	6.25	1.56
SERRATIA 2852	> 100	-	> 100
PSEUDOMONAS	> 100	> 100	> 100
PROVIDENCIA 2851	3.12	-	6.25
PROTEUS VULGARIS 2629	1.56	-	6.25
PROTEUS MORGANII 2834	6.25	-	6.25

The Shionogi group in Japan has been extremely active for many years in the synthesis of nuclear modified cephalosporins and they have devised a stereospecific synthesis for these analogues. Results of their experiments are shown in Table 3.

Table 3

ANTIBACTERIAL ACTIVITY (MIC) OF 1-OXACEPHEMS



ORGANISM	R$_1$	R$_2$	R$_1$	R$_2$
	 CH$_2$	0 Ac		
	X = S	X = 0	X = S	X = 0
STAPH. AUREUS 209P JC-1	0.05	0.013	0.1	0.025
*STAPH. AUREUS C14	0.2	0.1	0.4	0.4
STREP. PNEUMONIAE 1	0.1	0.025	0.1	0.025
E. COLI NIHJ JC2	6.3	0.8	0.4	0.05
KLEBSIELLA PNEUMONIAE	0.8	0.2	0.4	0.05
*KLEBSIELLA SP 363	> 100	> 100	> 100	> 100
PROTEUS MIRABILIS PR-4	3.1	0.4	0.8	0.1
PROTEUS VULGARIS CN329	50	50	0.8	0.1
ENTEROBACTER CLOACAE 233	> 100	> 100	3.1	1.4
SERRATIA MARCESCENS	> 100	> 100	50	6.25

*LACTAMASE PRODUCER

Firstly, there is no doubt that the 1-oxa derivatives are at least as potent as the parent thia compound. Most welcome is the fact that the increase in potency of the oxa analogue is greater with Gram – ve organisms than with Gram + ves, although the changes are quantitative rather than qualitative and the spectrum is not significantly broadened.

The replacement of S by O did not confer resistance to β lactamases.

The conclusion from the work on nuclear modification of cephalosporins was then that the microbiological spectrum could be retained, the potency against Gram – ves increased, but that lactamase stability was unchanged. Significantly, oral absorption has not been reported to be improved.

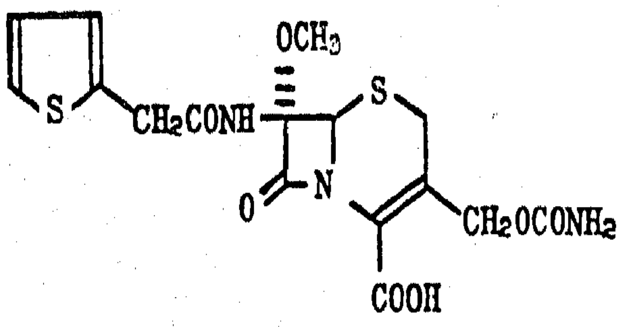
Thus, this elegant chemistry had produced little in the way of new molecules. However, it had led to the development of elegant and economic routes to make cephalosporins from penicillins and gave rise to one significant compound from Shionogi which I shall discuss later.

Nuclear and Side Chain Substitutions

Nuclear modification had not improved the lactamase stability of the cephalosporins. The solution to this problem came, instead, from substitutions, and in two ways.

Firstly, by the discovery by Merck Sharp & Dohme of the naturally occurring cephamycins. These are cephalosporins having an OMe substituent on the cephalosporin ring at the 7 position. From this discovery MSD developed the cephamycin derivative Cefoxitin which is highly resistant to lactamase attack by virtue of the 7-OMe group. (Figure 2).

The recognition that the 7-OMe group conferred lactamase resistance was taken up by Shionogi in relation to their oxa cephalosporins and they synthesised a large number of oxa cephamycins for investigation.



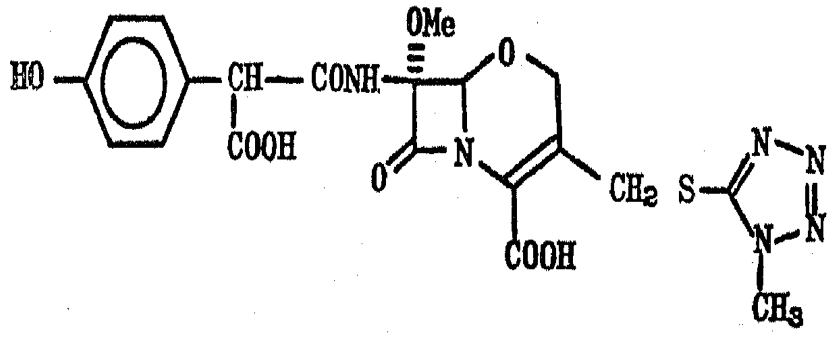
CEFOXITIN (MSD)

Fig. 2

The substance chosen for development by them was S 6059 which has the good Gram - ve activity associated with the oxa substitution, and is lactamase resistant by virtue of the 7-OMe group.

The structure of S 6059 is shown in Figure 3.

OXA-CEPHAMYCINS



S - 6059 (SHIONOGI)

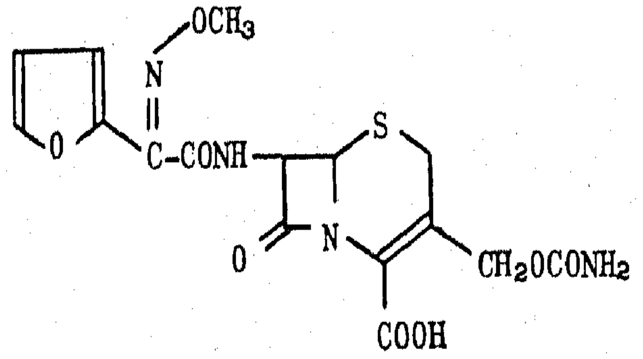
Fig. 3

As you can see it has the well known tetrazole thioether side chain in the 3 position — like Cefamandole — but unusually has an acidic 7 side chain. Not surprisingly, it is not orally absorbed.

More recent reports from Shionogi indicate that halogen substitution in the aromatic ring on the 7 side chain can give improved Gram +ve activity. Whether the high Gram - ve is retained is not clear — one might expect Gram - ve potency would be reduced by analogy with the S containing cephalosporins.

Another strategy for conferring lactamase stability on cephalosporins was discovered by Glaxo, who sought ways of adding the OMe group of cephamycin to the cephalosporin nucleus in a way that was chemically simple. One of these attempts added the OMe group not to the β lactam ring (as in Cefoxitin) but to the carbon adjacent to the amide carbon in the side chain via the syn-oxime.

The compound developed by Glaxo — Cefuroxime — is shown in Figure 4.

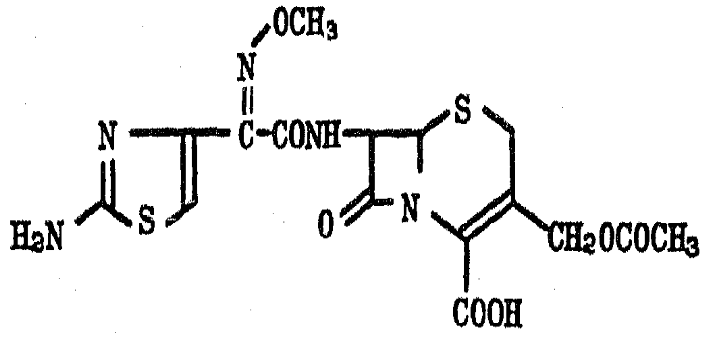


CEFUROXIME

Fig. 4

The ancestry of Cefuroxime is fairly obvious and, like Cefoxitin, Cefuroxime is extremely resistant to lactamase attack.

Not surprisingly the discovery of Cefuroxime prompted an intensive synthetic effort to make other syn-oximes, culminating in the synthesis of Cefotaxime by Roussel, whose structure is shown in Figure 5.



HR 756 CEFOTAXIME

Fig. 5

Cefotaxime has the methoximino side chain of Cefuroxime, making it lactamase resistant. It has the new aminothiazole substituent in the 7 position, which confers extraordinary potency on the structure, making it 10 times more potent against certain Gram-ve organisms. The 3 side chain is unchanged from that present in Cephalosporin C.

The activities of Cefotaxime (HR 756) and S-6059 are compared in Table 4.

It is seen that both compounds are extraordinarily potent, broad spectrum agents with very good activity against Gram-ves especially pseudomonas. Not only are both compounds resistant to β lactamase attack but they are inhibitors of lactamases.

However, neither is orally active, S-6059 is extremely expensive to make and HR 756, like all cephalosporins which retain the acetoxy methyl side chain from Cephalosporin C (such as Cephalothin), is metabolised to some extent with loss of activity. The metabolism of HR 756 is summarised in Figure 6.

The desacetyl derivative is first formed by hydrolysis of the ester, the free alcohol then forming a lactone with the 4-COOH group. The desacetyl cefotaxime is still a potent antibiotic, but the lactone is devoid of significant activity.

In order to minimise the loss of antibacterial activity associated with the metabolism of Cefotaxime, it would be theoretically possible to either:

(a) remove the sensitive acetoxy methyl side chain from Cefotaxime or

(b) replace it with a non-metabolisable side chain.

Table 4

COMPARATIVE ACTIVITY OF HR 756 AND S-6059

ORGANISM	NO.	MIC RANGE		90% MIC	
		HR	S	HR	S
S. AUREUS	18	0.4 - 25	3.1 - 25	3.1	6.2
S. EPIDERMIS	16	0.2 - 25	0.4 ->100	6.2	50
STREP. PYOGENES	15	0.01 - 0.2	0.8 - 3.1	0.1	1.6
STREP. AGALACTIAE	20	0.01 - 0.2	0.4 - 3.1	0.1	3.1
STREP. VIRIDANS	5	0.05 - 0.8	0.4 ->100	0.4	>100
STREP. FAECALIS	13	1.6 ->100	3.1 ->100	>100	>100
STREP. PNEUMONIAE	10	0.01 - 0.2	0.2 - 6.1	0.1	3.1
H. INFLUENZAE	12	-	0.1 - 3.1	-	1.6
N. GONORRHOEAE	13	0.01 - 0.4	0.4 - 1.6	0.4	0.8
E. COLI	40	0.02 - 3.1	0.02 -12.5	0.4	0.1
KLEBS. PNEUMONIAE	37	0.02 - 0.4	0.05 -12.5	0.4	0.8
ENT. AEROGENES	16	0.05 ->100	0.05 - 3.1	0.4	3.1
ENT. CLOACAE	18	0.05 - 0.8	0.05 - 25	0.4	12.5
PROTEUS MIRABILIS	34	0.02 - 3.1	0.05 - 1.6	0.1	0.1
PROTEUS VULGARIS	11	12.5 ->100	0.1 - 25	>100	3.1
PROTEUS RETTGERI	13	0.01 - 1.6	0.1 - 25	1.6	12.5
M.MORGANII	15	0.01 - 1.6	0.1 - 0.2	1.6	0.1
PROVIDENCIA	10	0.05 - 3.1	0.05 - 0.1	0.8	0.1
CITROBACTER	10	0.02 - 0.1	0.02 - 0.2	0.1	0.2
SERRATIA	34	0.1 - 50	0.05 - 50	50	50
SALMONELLA	17	0.02 - 0.2	0.02 - 1.6	0.1	1.6
SHIGELLA	18	0.05 - 1.6	0.05 - 0.4	0.8	0.2
ACINETOBACTER	11	1.6 ->100	1.6 ->100	>100	>100
B.FRAGILIS	11	12.5 ->100	0.05 - 100	>100	50
BACTERIOIDES SP	21	3.1 ->100	0.05 ->100	>100	>100
PS.AERUGINOSA	79	0.4 ->100	6.2 ->100	>100	>100

REF. NEU, ANTIMIC. AGENTS 16, 141 (1979)

METABOLISM OF CEFOTAXIME

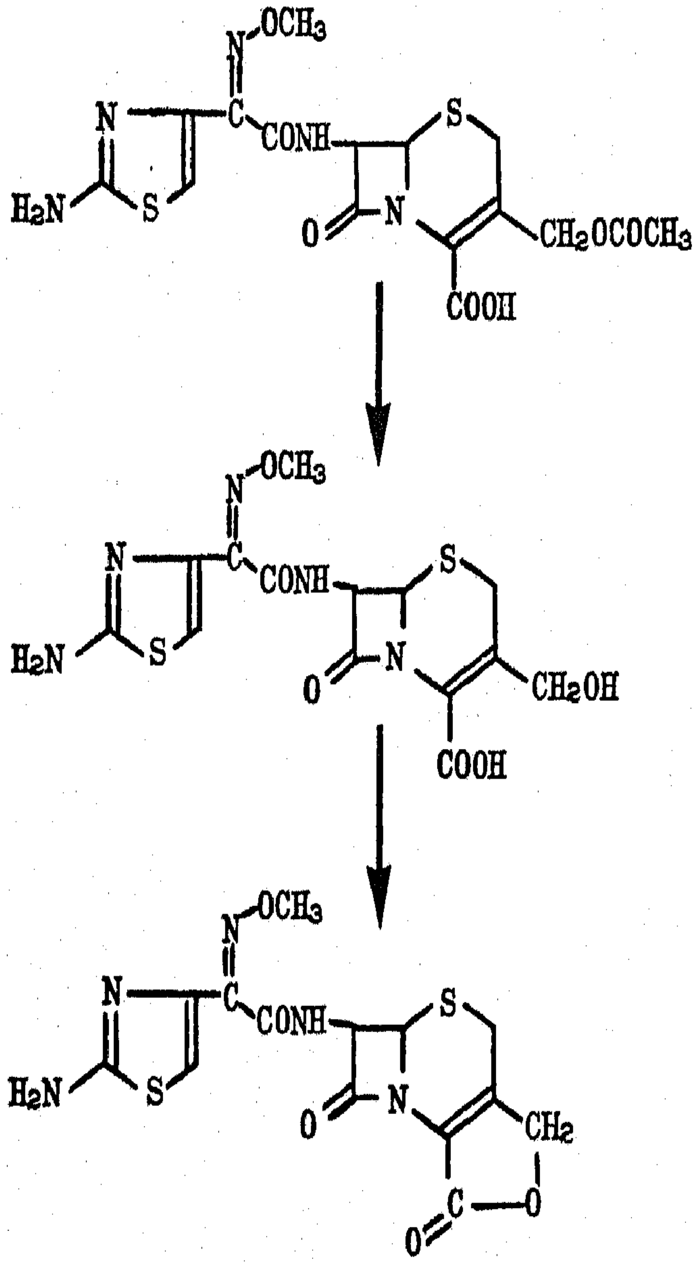
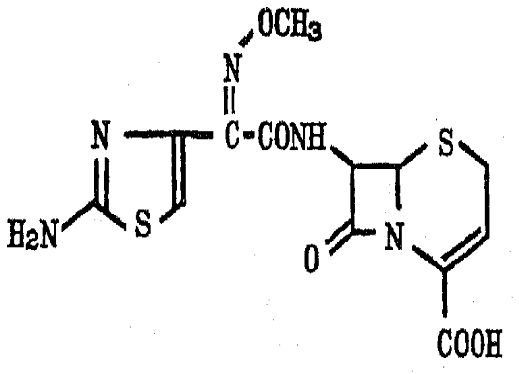


Fig. 6

In practice, both strategies have been adopted.

Fujisawa, in their compound FK 749, have removed the entire 3 side chain from Cefotaxime as shown in Figure 7.

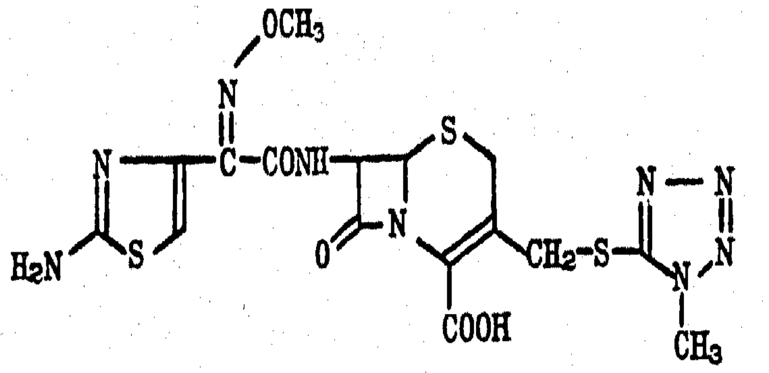


FK 749 (FUJISAWA)

Fig. 7

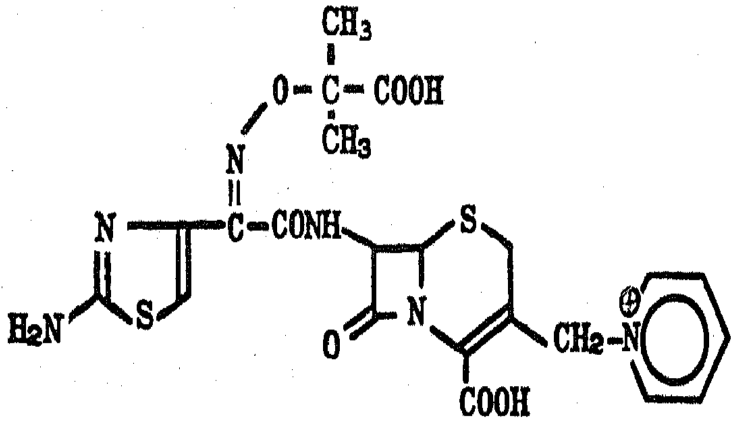
Takeda and Glaxo, on the other hand, have replaced the labile 3-acetoxymethyl side chain with metabolically inert substituents. Takeda have used the well known Cefamandole side chain N-methyl tetrazole thio ether in their derivative SCE 1365, (Fig. 8) whereas Glaxo have returned to the 3-pyridyl side chain which they first used in Cephaloridine many years ago.

The structure of the latest Glaxo compound GR 20263 is also shown in the same figure.



SCE 1365 (TAKEDA)

Fig. 8


CH3
O
C
COOH
CH3
N
N
C
CONH
S
H2N
S
O
N
COOH
CH2
N

GR 20263 (GLAXO)

Fig. 8 (*contd.*)

It will be seen that the bulk of the syn-oxime group has also been increased, but the important amino-thiazole ring of Cefotaxime is retained. This compound is reported to have better pseudomonas activity than Cefotaxime, and is naturally highly resistant to lactamase attack. It is not as active as Cefotaxime against Gram +ve organisms.

To summarise this part of my talk, the development of S-6059, Cefotaxime, FK 749, and GR 20263 represents a significant collection of highly potent, lactamase resistant, Gram – ve active cephalosporins.

Orally Active Compounds

The general lack of oral absorption of the cephalosporins has long been a puzzle and an impediment. There is no doubt that an orally active β-lactam with cephalosporin like properties is the major therapeutic goal of β-lactam research today. Why is it that orally absorbed penicillins are easily found, whereas orally absorbed cephalosporins are the exceptions that prove the rule?

Figure 9 shows the orally absorbed cephalosporins. It will be noticed immediately that they all have a basic ampicillin like side chain in the 7 position with a free amino group.

ORALLY ACTIVE CEPHALOSPORINS

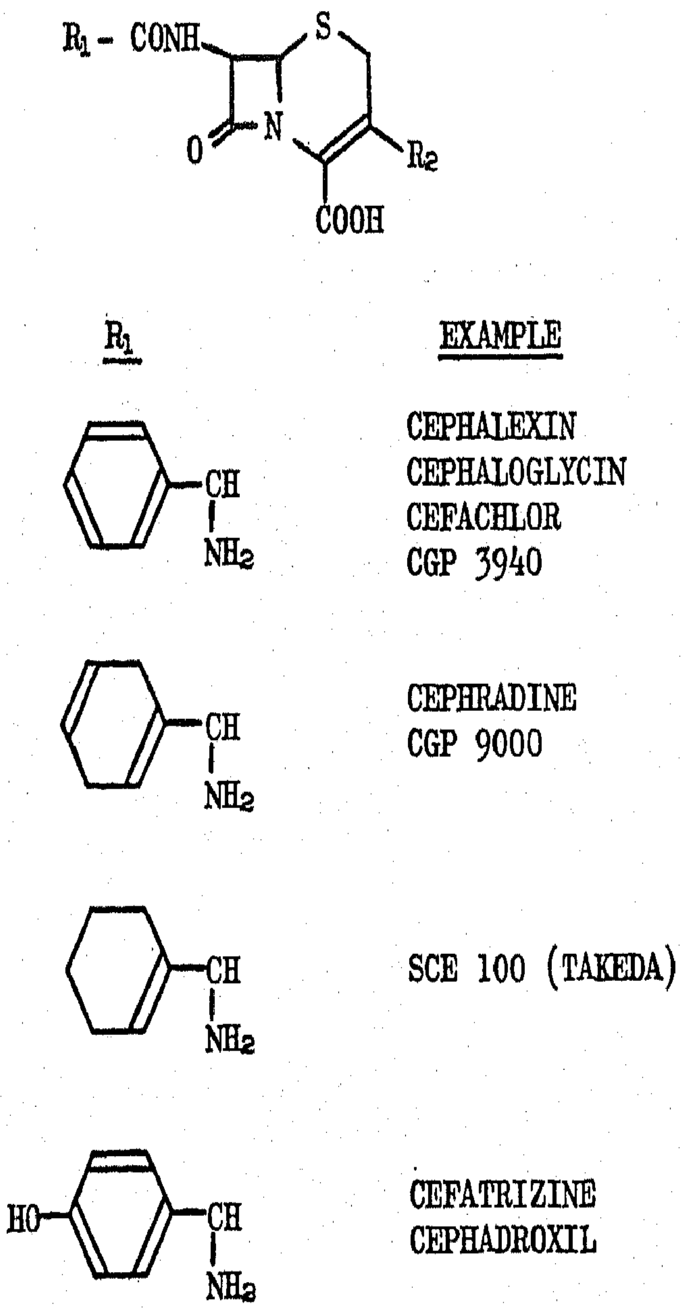

R1 - CONH
S
O
N
R2
COOH
R1
EXAMPLE
CH
NH2
CEPHALEXIN
CEPHALOGLYCIN
CEFACHLOR
CGP 3940
CH
NH2
CEPHRADINE
CGP 9000
CH
NH2
SCE 100 (TAKEDA)
HO
CH
NH2
CEFATRIZINE
CEPHADROXIL

Fig. 9

The simplest explanation is that cephalosporins in general are not absorbed but examples possessing this particular phenylglycine amide side chain are taken up by an active transport process in the gut and are, thereby, absorbed. Unfortunately, active transport processes tend to be limited, both in capacity and location in the gut, and I am afraid that, if this hypothesis is correct, then the outlook for achieving orally absorbed active cephalosporins is not very encouraging. This is confirmed by the fact that, whilst many pro-drug esters of penicillins exist, none have been found for cephalosporins.

Although the variation at the 7 position is extremely restricted, considerable variation at the 3 position is possible with retention of oral activity as shown in Table 5.

The last column compares the oral and subcutaneous doses giving equal efficacy (i.e. 50% protection). Compounds with a PO/SC ratio of 1 or less are orally active. It can be seen that a range of 3-substituted analogues are active.

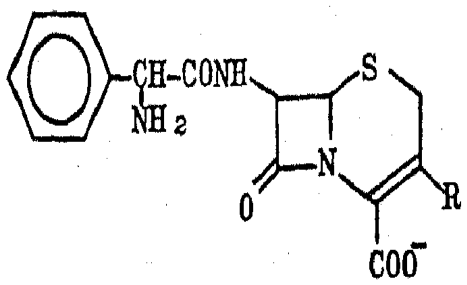
Ciba-Geigy have made a very extensive study of 3-substituted cephalosporins with the phenylglycine amide side chain. The optimal electron withdrawing substituent at the 3 position, which had adequate biological activity and chemical stability, was the OMe derivative (CGP-9000) which is under development as an orally active cephalosporin (Figure 9).

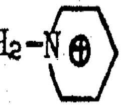
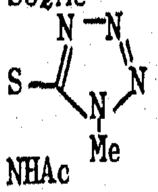
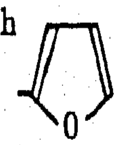
It may be asked, are the oxa cephalosporins with the phenylglycyl amide side chain better absorbed than the normal thia analogue? The answer is no. The phenylglycyl oxacephalosporins are very unstable due to aminolysis of the β-lactam ring by the free amino group.

Finally, since it is known that in the cephalosporins – such as Cefotaxime, FK 749, SCE-1365 — with the aminothiazole syn-methoximino side chain in the 7 position, wide variations at the 3 position are possible with full retention of activity, attention is being given to finding substituents to promote absorption at the 3 position on Cefotaxime.

Table 5

ORAL ABSORPTION OF CEPHALOSPORINS



R	MIC		ED₅₀ STAPH.AUREUS		
	S.AUREUS	E.COLI	P.O.	S.C.	PO/SC
CH_3 (CEPHALEXIN)	1.6	12.5	7.5	12	0.6
CH_2OAc (CEPHALOGLYCIN)	3.1	6.2	8	6	1.3
CH_2-N⊕  (CEPHALORIDIN)	0.05	3.1	1.7	0.17	10
H	2.5	3.5	2.7	16	0.2
COOMe	30	>100	-	-	-
COOH	3.5	>100	>100	>100	-
C ≡ N	0.4	>100	100	30	3.3
CHF_2	>100	>100	>100	>100	-
OCH_2Ph	0.05	30	15	30	0.5
O nBut	0.4	30	20	20	1.0
OEt	0.4	5	10	14	0.7
OMe	3.1	3.1	7.5	10	0.7
SMe	0.3	3	4	3	1.3
SPh	10	>100	>100	10	>10
SCH_2COOMe	60	>100	7	12	0.6
SO_2Me	>100	>100	>100	>100	-
	60	>100	>100	80	> 1
NHAc	4	>100	>100	35	> 3
$NHCOCH_2Ph$	1	>100	>100	55	> 2
NHCONHCO 	0.8	25	150	90	1.7
CH_2COOMe	3	10	3	6	0.5

Modification of Penicillins

The modifications that have been described above for cephalosporins, and which have led to compounds with increased potency, Gram–ve activity and lactamase resistance, have been nowhere near as successful with the penicillins. This is a disappointment since the penicillins have the overriding advantages of being both cheap and usually orally absorbed. Unfortunately, they only possess one side chain for structural modification which has limited the improvement possible by semi-synthetic modification.

So far as nuclear modifications are concerned, the replacement of S by 0, in contrast to the cephalosporins, results in a significant loss of activity. As with the cephalosporins, the change from S to 0 does not confer lactamase stability on the penicillins.

The use of the syn-oxime to confer lactamase stability and improve Gram-ve activity in the cephalosporins, does not seem to have been successful with the penicillins and I know of no publications on syn-oxime penicillins. Perhaps, the counterparts of the syn-oxime cephalosporins are the ureido penicillins, such as Mezlocillin and Azlocillin, which do possess Gram-ve activity but which, unfortunately, like Ampicillin are readily hydrolysed by β - lactamase enzymes.

Also, oral activity has been sacrificed in the ureido penicillins. The addition of the 6-0Me group to the penicillin nucleus to form the penicillin equivalent of the cephamycins results in a significant loss of activity, although lactamase stability is improved.

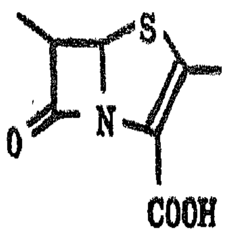
The most successful strategies in achieving lactamase resistance in the penicillin field have been:

1. the replacement of the 3-carboxyl group by a tetrazole moiety, and
2. the addition of a broad spectrum lactamase inhibitor such as Clavulanic acid.

Non Penicillin/Cephalosporin β – lactams

I would now like to turn to β - lactams, which are non-classical i.e. are neither penicillins, nor cephalosporins.

An obvious ring-structure is the penem ring (Fig. 10), which has features of both the cephalosporins and penicillins.

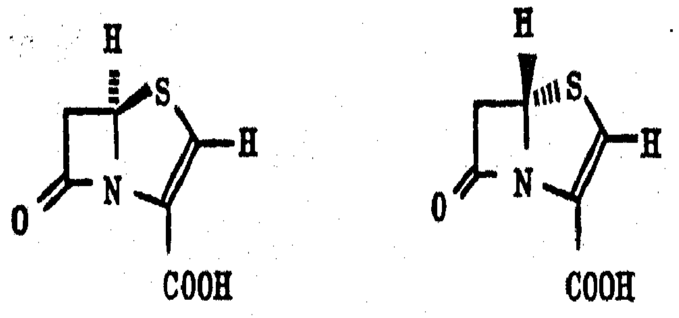


PENEM

Fig. 10

Unfortunately, the basic penem ring structure with a 6 amino substituent (benzyl substituted) was less potent than either the corresponding penicillin or cephalosporin. Quite unexpectedly, however, the Woodward group at Ciba-Geigy found that the *unsubstituted* penem (i.e. lacking the 6 amino group) was both highly active *in vitro* and was resistant to lactamase attack. The stereochemical requirement for activity was similar to that known for the penicillins and cephalosporins. These revelations obviously stimulated interest in penems lacking the 6 - amino function normally associated with the penicillins (Figure 11).

BIOLOGICAL ACTIVITY OF PENEMS



ACTIVE **INACTIVE**

Fig. 11

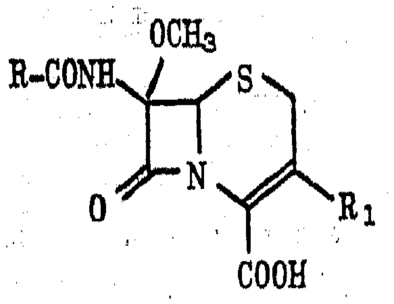
It was about this time that two other non-classical β - lactam ring types were announced. Like cephamycins, these were discovered in Streptomyces fermentations rather than in the organisms usually associated with β - lactam formation.

The three streptomyces derived β - lactams are shown in Figure 12.

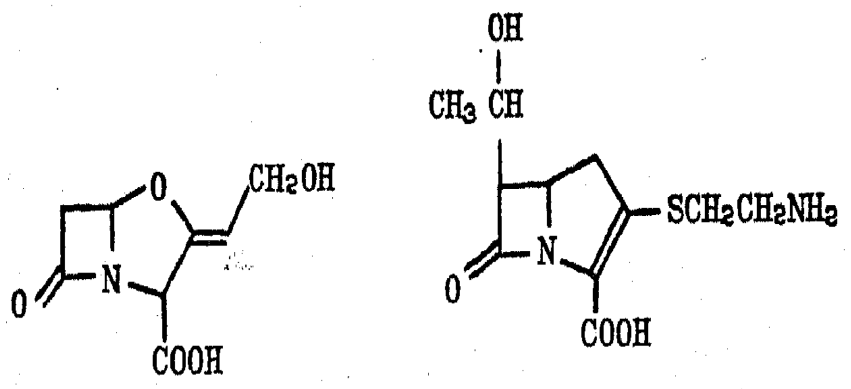
Thienamycin is an extremely potent antibiotic and will be discussed shortly. At this point I would emphasise only that it has a carbapenem ring structure, and again lacks a 6-amino substituent.

The third β - lactam is Clavulanic acid, an oxape*nam*, which again lacks a 6 amino substituent. Clavulanic acid is an extremely potent inhibitor of β - lactamase although it has only weak Gram +ve antibiotic activity.

NEWER β LACTAMS



CEPHAMYCINS



CLAVULANIC ACID THIENAMYCIN

Fig. 12

Beecham have reported the synthesis of the oxape*nem* derivative of Clavulanic acid but no biological data was given. By analogy with the oxa cephalosporins (but not oxa penicillins) one might have hoped for biological activity.

These results all point to future interest in the area of oxa, carba, and thia penems, which lack the 6-amino group associated with the penicillins.

Thienamycin

I should like to finish by talking about Thienamycin.

The structure of Thienamycin is shown in Figure 13.

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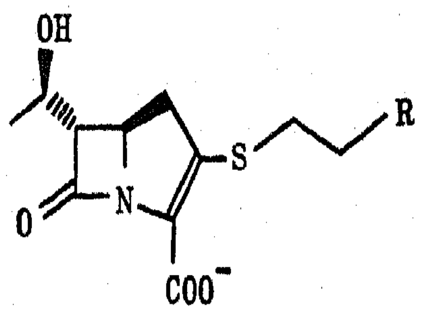
THIENAMYCIN



R	COMPOUND
NH_2	THIENAMYCIN
NHCH = NH	MK 0787
$NH-\underset{CH_3}{C}=NH$	N-ACETIMIDOYL THIENAMYCIN

Fig. 13

The first point to note is that, not only is the 6-amino group absent, but the hydroxyethyl side chain is in the *trans* configuration, in contrast to the *cis* configuration of the penicillins. Although the *cis* (epi) thienamycin derivative has been isolated, it is less active, particularly against pseudomonas, as compared to Thienamycin itself and there seems no doubt that the preferred configuration for biological activity, in Thienamycin is different from that for the penicillins, cephalosporins and penems. Indeed, it is likely that the lactamase resistance of Thienamycin is due to its 'non-classical' stereochemistry at the 6 position.

Thienamycin is an extremely potent broad spectrum antibiotic. Unfortunately, it is chemically quite unstable, undergoing a bimolecular condensation reaction involving acetylation of the free amino group on the thio alkylamino side chain. In order to prevent this, an obvious strategy was to substitute the free amino group on the molecule to make it resistant to attack. Unfortunately, it was known that the naturally occurring N-acetyl thienamycin was less active especially against Gram-ves than Thienamycin, although chemically it was more stable than the parent molecule.

Merck, therefore, adopted the strategy of increasing the basicity of the amino group by converting it to an amidine (or guanidine) which it was hoped would achieve chemical stabilisation and retain Gram - ve activity.

Of the two derivatives reported by Merck, the N-formimidoyl and N-acetimidoyl, the activity of the former (MK 0787) is shown in Table 6.

Table 6

COMPARATIVE ACTIVITY OF THIENAMYCINS

ORGANISM	THIENAMYCIN		MK 0787	
	MIC	ED_{50}	MIC	ED_{50}
S.AUREUS	0.01	0.06	0.02	0.26
E. COLI	0.08	2.5	0.16	4.2
Pr. MIRABILIS	5.0	1.0	10.0	15.1
E. CLOACAE	0.63	0.65	0.63	1.56
Ps. AERUGINOSA	2.5	1.2	5.0	2.4
B. FRAGILIS	0.2	-	0.4	-

It can be seen that there is little loss of biological activity, and chemical stability is completely satisfactory. Unfortunately, no data have been reported on oral absorption, although it must be anticipated that the increased basicity will reduce the level of oral absorption below an acceptable level.

Thienamycin is but one example of the new class of β-lactams. The Olivanic acids of which many members have now been isolated and identified are a second example. Some are lactamase inhibitors and some are antibacterials.

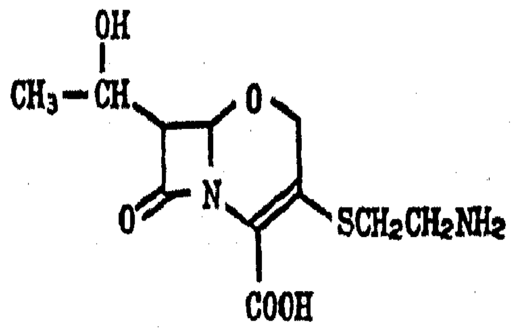
Cephems

The discussion above has indicated the lively interest in oxa, carba, and thia penems arising from the activities associated with the unsubstituted penem ring itself, Thienamycin and Clavulanic acid. However, this interest, it must be remembered, relates to structures lacking a 6-amino substituent on the β-lactam ring.

It was a natural question to ask what is the activity of oxa, carba and thia cephems lacking the normal 7-amino substituent.

Unfortunately, little published information is available to answer this question, and such compounds are not easily synthesised. However, one pointer is a report of the synthesis of an oxa cephalosporin with a 7-hydroxyethyl and 3-(amino thioethanol) side chain, both of which correspond to the side chains of Thienamycin

(Figure 14). This oxa-cephalosporin is claimed to have activity against Gram +ve organisms including *Pseudomonas aeruginosa*. Clearly, cephems lacking the 7 amino substituent may also provide a fertile area for future investigation.



US PATENT

Fig. 14

Conclusion

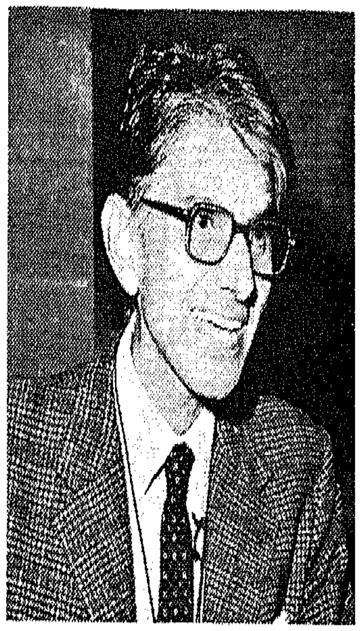
I hope I have demonstrated in this talk that the field of β-lactams represents an almost infinite and fascinating challenge to the seeker for new and better antibiotics. I have tried in my talk to bridge the gap between chemistry and microbiology, and show the paths we have travelled to reach our present position. I hope I have shown you, too, how there are challenges still to be met, notably in the field of orally active agents, and how the synthetic chemist and the natural product chemist have in the past combined their talents to produce new molecules of dramatic effectiveness. I am confident that we will continue to see equally impressive advances in the future.

PR

NEW APPROACHES TO THE GENETICS OF ANTIBIOTIC PRODUCING ORGANISMS

by

Prof. O. Siddiqi



Professor Obaid Siddiqi took his M.Sc. degree from Aligarh University and obtained his Ph.D. degree from the University of Glasgow with Prof. G. Pontecorvo.

He worked at Yale University, U.S.A. and the Laboratory of Molecular Biology at Cambridge before joining the Tata Institute of Fundamental Research in Bombay in 1962.

Prof. Siddiqi's contributions are in the area of molecular genetics and the genetic aspect of Neurobiology. He was a visiting Professor of Biophysics at the Massachusetts Institute of Technology and Gosney Fellow in Behavioural Genetics at Cal-Tech. A Fellow of the Indian National Sciences Academy and also of the Indian Academy of Science, he was awarded the Bhatnagar Prize for Biological Sciences in 1976.

Prof. Siddiqi is presently Prof. of Molecular Biology at the Tata Institute of Fundamental Research and Head of it's Molecular Biology Group.

Mr. Chairman, Ladies & Gentlemen,

I must first thank you and the organizers of the Symposium for calling me here. Believe me, I was most surprised to be invited to speak at this meeting. So far as I know, I have never worked with antibiotics. Therefore, the only justification that I could think of for being here is that 20 years ago when I started working on genetics, I started with an organism, *Aspergillus*, which perhaps will just nominally qualify to be an antibiotic producer. Very soon I diverted from that organism and began to work with *E. coli* and its phages and, a few years ago, I stopped doing even that. I, therefore, feel quite incompetent to make any substantive remark about the genetics of antibiotic production. But I am going to take this opportunity of making some very general remarks on what genetics, as we know it today, might have to do with antibiotic production. I make these remarks not really as a specialist but as one who is interested in genetics generally and genetics of microorganisms in particular and I address these remarks especially to my colleagues in the field of antibiotics who come from Biochemistry and Microbiology.

Microbial genetics is now about 30 years old. Before this it was generally thought that microorganisms have no genetics. At least they have no sexual reproduction. There was a belief that microorganisms continuously adapt to the environment in which they grow and 25 years ago it took some very clever experiments and a great deal of persuasion by people like Luria, Delbruck and Lwoff to convince everyone that microorganisms mutate more or less in exactly the same way as other animals and plants do. Since then we have gone a great length. Everyone is now convinced that microorganisms have perfectly garden variety genetics. And, in fact, in the past 30 years, such a vast store of knowledge of genetics of microorganisms has accumulated that it has revolutionized the ideas of not only the genetics of microorganisms but of biology in general. One would, therefore, be very justified in asking what has all this done to the production of antibiotics. And I am afraid the honest answer must be, not a great deal. Our knowledge of the biochemical pathways of antibiotic production and its genetic control is still fragmentary and quite incomplete. It has been pointed out by, I believe, David Hopwood, that microbiological industry has not done to microbial genetics what plant breeding did to plant genetics. It has certainly not very greatly stimulated a deeper understanding of the genetics of antibiotic production. What are the reasons for this? First, geneticists have chosen for their study of synthesis and catabolism, organisms like *E. coli*, *Salmonella*, *Neurospora*, *Aspergillus*, *Saccharomyces* for, primarily, the technical ease of working with these organisms and, then, for the very sound reason of concentrating all efforts on one or two organisms so that we can understand them fully and completely. This has been fully justified by the results. But all microorganisms are not alike. And there are certain things which microorganisms like Actinomyces and Bacilli have which others don't.

And these still remain to be learnt. There is a somewhat general reason for this. Geneticists and biochemists in the past 20 years or so have concentrated on the bio-chemistry of what are called primary metabolites; these are things like amino acids which make protein and bases which make nucleic acids. These things are important and essential and for the very reason that all organisms have them in the same way and synthesize them by somewhat similar pathways. Secondary metabolites are often special to microorganisms. They are also very often synthesized under special conditions, for instance, at the time of sporulation, before sporulation or after sporulation and it turns out that many antibiotics are related to such secondary metabolism. The regulation of such metabolites, for the very reason that they are synthesized under special conditions of growth, is some what hard to study. Certainly not so easy to study as that of an organism, as it is something which is made over the entire life cycle of a cellular organism not growing logarithmically. It is for these reasons that, by and large, academic biologists, who have primarily been concerned with the understanding of basic mechanisms, have kept away from such metabolites and such organisms. Very fortunately, though, there is at least one, what one might call academic, organism which is related to the major groups of antibiotic producing organisms. I hardly have to tell you that even though the groups of microorganisms are many, there are indeed only 3 major groups which are concerned with antibiotics. It turns out that, at least, we have one representative of each group for which genetics has been very highly developed. *Aspergillus nidulans* for the filamentous fungi, *Streptomyces coelicolor* for Actinomycetes and *Bacillus subtilis* among bacilli. I can, therefore, hope that at least these 3 organisms will act as something like the existence theorem of genetics for antibiotic production.

I will spend a few minutes talking about what we know of the genetic system in groups of microorganisms which are important in antibiotic production. What do I mean by genetic system? In order to carry out definitive analysis, either genetic or biochemical, with the help of genetics, one needs to be able to carry out a few operations. For instance, one must have genetic transfer, a system in which genes or DNA from one cell can be transferred to another. Such transfer leads to the existence of more than one complement of genes in the same cell, heterokaryosis or formation of diploids or partial diploids (merodiploids). If one can do this, one can study interactions between genes, one can carry out complementation tests which allow one to decide whether two mutations are in the same gene, whether they affect the same function, or they in are different genes or the action of one gene on another which is the study of genetic regulation. In order to carry out any one of these classes of experiments, one must, therefore, have good effective systems of genetic transfers. And, lastly, in order to have the analysis of genetics in its full force, one must have re-combination. Re-combination does not do merely what a breeder always expects it to do, that is, give a new combination of genes. But it also enables you to learn a great deal about genes, to map the genes i.e. their positions on the chromosomes, to determine the final structures of genes which are often very important. For instance, this is one of the ways in which one can gain very precise information about the existence of complex multi-enzyme systems determined by the genetic structure and a variety of other useful information from the point of view of understanding a biosynthetic pathway.

Let us see, first of all, what sort of genetic system exists in the groups of microorganisms in which we are interested. First, filamentous fungi. Of about 3000 or so known or reportedly known antibiotics which exist, about one fourth come from fungi. The group which produces a great majority of these are Aspergillaceae, to which belong *Aspergillus* and *Penicillium* and some 10 or so antibiotics out of this last group are in fact commercially important. The interesting thing is that several of these commercially important antibiotics out of this group are in fact made by more than one organism. For fungi, one organism for which the conventional sexual genetics is fully developed is *Aspergillus nidulans*. With this organism one can do the entire gamut of genetic operations: breeding, re-combination, mapping and so on and so forth. Primarily, as a result of early work on this organism, novel systems of genetic analysis were developed which are now called parasexual genetics.

This, in fact, is applicable to a much larger group of fungi including Penicillium and a number of other Aspergillus species. In the parasexual cycle, one can make heterokaryons of different strains leading ultimately to diploidisation. The technique for making diploids, at least in fungi, which have uninuclear conidia was developed as early as 1950 by Alan Roper. Once one has diploids one can have in diploids mitotic crossing over which overcomes the deficiency of conventional sexual reproduction if it does not happen to exist in the fungus. And one can use this to do a variety of sophisticated genetic analyses which one does normally on the sexual side.

The second group of organisms, and perhaps the most important from the point of view of antibiotic production, are the Actinomycetes. This group yields the largest number of antibiotics, over 2000, and about 70 of these are commercially produced. These include Chloramphenicol, Streptomycin, Erythromycin, Kanamycin, Tetracyclines and so on. Important species in this group which are amenable to genetic analysis are the streptomyces, especially *Streptomyces coelicolor*, whose genetics has been very successfully developed by David Hopwood and it is believed that many other streptomyces would have similar genetic mechanisms as you know, for example, species like Nocardia are probably easily amenable to genetic analysis. In streptomyces the method of genetic analysis is very similar to that in *E. coli*. It is some form of conjugation, although the detailed mechanism of transfer is not so clearly understood as in *E. coli*. It is something which is very akin to conjugation. Using this conjugation a precise map of streptomyces has been made and one can do operations which are used in doing conventional diploid tests. There are also at least some reports of transduction in streptomyces, although I do not know whether these are common. In mycobacteria, there is transduction. It has, in recent years, been developed in more than one laboratory and, finally, in thermoactinomycetes there is transformation. There are species of thermoactinomycetes which show a very high frequency of transformation, almost in the order of 10^{-3}. And finally, we come to the eubacteriaceae to which the common enteric bacteria belong. These produce about 300 antibiotics and the most important organisms among these bacteria from the point of view of antibiotic production are bacilli and pseudomonas. Among bacilli, there are several species and these constitute about half of the antibiotic producers. As I have already said, the genetic analysis of *Bacillus subtilis* by transformation is a very highly developed field and a great deal is known about the genetic organisation of *Bacillus subtilis* chromosomes. Subsequently, transduction for *Bacillus subtilis* was developed and has added very greatly to successful analysis of the organisation of the *subtilis* chromosome. The genetics of pseudomonas is developing very fast. This was the organism in which Gunsalus and many of his students and colleagues made important contributions in developing and using plasmid genetics as a way of manipulating the organism and it is clear that it has become one of the most successful organisms for doing plasmid genetics. Of course, there is a vast amount of work with enteric eubacteriaceae like *E. coli*, salmonella and their species and I will not talk about it because, by and large, it is peripheral to antibiotic production. So, in summary, I can say that as far as the genetic systems go, it is clear that there are no intrinsic hurdles to genetic manipulations in antibiotic producing organisms.

I will now turn for a minute to a point which is more of a physiological or biochemical nature. Genetics is not merely, or should not merely be looked upon as, a way of making an organism produce more of something. It is more than that. It is a method of understanding how the organism produces something. Unfortunately, this power of genetics has not been utilized to its full extent in understanding production of antibiotics by microorganisms. There could be many reasons for this. It turns out, for instance, that mutations that effect antibiotic production very often have pleotropic effects, that is, complex effects. They do many things to the organism often leading to a decline of its synthetic ability. Geneticists working with

microorganisms inevitably use auxotrophic mutations as genetic markers because you can get many of them, many kinds of them. It turns out that most often or very often such auxotrophic mutations decrease the yields. These are phenomena, these are things, which keep off people who are primarily interested or overtly interested all the time in increasing yields and who work with such organisms. At least that is my suspicion. There is also, what seems to me, a more fundamental reason for the difficulty of working on the regulation biochemistry of antibiotic producing organisms. As I said before when one is dealing with primary metabolites the pathways of synthesis of primary metabolites are unique pathways. That is, there is one and only one way in which a series of transformations leads to the formation of a unique class of molecules, and if you block or cut off any enzyme in this pathway, you block the synthesis of one of these steps, and the precursor begins to accumulate. It is very rarely that the precursor is shunted off to some other pathway and effects the metabolism of the cell as a whole.

By and large, this does not seem to be true of secondary metabolites. Secondary metabolites can be transformed in more than one way, as a rule in several ways. As a result of this, one is not dealing with a well defined single pathway but many alternative pathways and there exists what has been described as a metabolic grid and disturbing this grid at one place creates effects in many other places. It is this which makes the understanding of the biochemical network of syntheses which are related to antibiotic syntheses a more difficult one to manipulate and perhaps acts as a deterrent. But one must point out that as a deterrent in using genetic analysis to understand regulation of metabolism. I must, however, point out that there are outstanding exceptions to this statement, the most outstanding being the study of the synthesis or biosynthesis of penicillin and cephalosporin. In both these cases mutants have been very effectively utilised in reconstructing the biosynthetic pathways and understanding the regulation of biosynthetic pathways for these two antibiotics.

Secondly, antibiotic production is likely to be controlled by many genes. This is true of many things but particularly so when one is concerned with increasing or decreasing the amount of antibiotic production. And, therefore, genetics is likely to be complex, it is likely to be quantitative genetics very often. All commercial strains of penicillin that are nowadays used are all derived from the Wisconsin family isolated between 1946 and 1956 in the Botany Department of the University of Wisconsin. Subsequently, these strains that were produced there and they all had high yields ranging from a factor of five or so above the normal production, these strains spread to various places in the world where they have been subsequently improved. These strains were used in earlier years to try and gain some insight into the genetics of the control of quantity of production. For instance, diploids were made between different strains to see what the levels of enzymes in the diploids were and the results were quite forbiddingly complicated. There were some strains where, when one made diploids, the level of the enzyme in the diploids was the same as that in the two parents but when other diploids were made the level in fact fell down in the diploids, below the levels of the parental strains from which the diploids were made. So, the study at least underlined the complexity of genetic control of quantity of antibiotic production and the only moral one can draw is that if one wants to understand the regulation of antibiotic synthesis, genetic regulation of antibiotic synthesis, then one should be able to study it with much greater determination.

I will take now a few minutes to talk about the prospective application of recombinant DNA technique to antibiotic production. This is something about which I am not going to say very much although the title of my talk may have given the impression that I am going to talk more about it. I will say something again about it more in the way of general remarks than describing to you all the good things that recombinant DNA research can do.

First, let me say a few words about the methodology of recombinant DNA. Primarily, this methodology has arisen from a fusion of plasmid genetics and the enzymology of DNA. Already, before DNA enzymes were used in manipulating the structure of DNA or synthesizing DNA of the kind one wants, a great deal was known about the multiplication and transmission of plasmids. The new thing that was added was the discovery of a set of enzymes which can most simply be described as scissors and glues or scissors and pastes. These enzymes were restriction enzymes and various ligases. They can be used to cut DNA into pieces of desired lengths and, if you like, at places where you want to cut the DNA and then these pieces can be joined to each other. One can, therefore, in short, make DNA by taking DNA from different sources and piecing them together. The methodology of handling DNA, manipulating it and inserting DNA into small plasmids which are now called cloning vehicles or cloning plasmids, has developed extremely rapidly in the past few years and there are now so many elegant ways of doing this that it goes beyond the scope of my intentions and time here to describe these two.

So let me just say that it is very possible now, without too much trouble, to take a fairly complex sample of DNA, not just a DNA from simple organisms like unicellular bacteria or phages or less diverse collections of genes, but DNA from fairly complex organisms, like insects, like Drosophila, and in fact put all the DNA of an organism in small bits into plasmids carried by bacteria, one by one, and one can then have a random collection of bacterial strains which between them carry all the genes of another organism and this is no fiction because this for instance has already been done with Drosophila. Such collections have been, I believe Walter Gehring in Switzerland first did it, called gene banks. People in America call them gene pools, or you can chose any name you like. For the Drosophila DNA, there are at least four laboratories which have such banks, complete collections of the Drosophila genes carried on plasmids in *coli* chromosomes. Therefore, it is just a matter of time and effort for anyone who is interested in any particular gene, to fish it out of the bank and do what he likes with it. Clearly, these operations can be carried out with heterogenous DNA, DNA which is sufficiently heterogenous, certainly at least two orders of magnitude more heterogenous than the DNA of most microorganisms. They should be much easier to carry out with any other microorganisms. So I imagine that, technically, the easiest thing in future to do would be to take a known piece of DNA and put it into any other microorganisms you like. What can it do for us? Well, in principle, the potential application of recombinant DNA technique eliminates the species barrier for our purpose. We can therefore make microorganisms synthesize for us anything that we want them to make, by putting in appropriate genetic information. This has already very elegantly been illustrated by the synthesis of strains which carry genes for Somatostatin. There you can get cultures of bacteria to make amounts of Somatostatin which will take millions of sheep to make. When one does this one encounters difficulties and the difficulties are very nicely illustrated by the case of Insulin. Genes for Somatostatins were inserted in *coli* and it worked beautifully. An elegant trick was used. Somatostatin was inserted in the lactose operon, and the control elements of the lactose operon were in fact used to make the bacteria produce large amounts of Somatostatin. However, when similar tricks were tried with Insulin it did not seem to have worked because there are at least two laboratories which have succeeded in introducing the rodent Insulin gene on to the bacterial plasmids. We know that the Insulin gene is there because we can grow these bacteria on the plate and by immunological tests show that the gene for Insulin is there and that it is expressing itself but the amount of Insulin it makes is very little, not of any practical use. So this merely underlines the problem that one does not simply have to put the gene in but one has to take care of the milieu in which the gene has to function. Well, for my purpose this makes a much more important point. Most of the applications of classical genetic technology to the microbial industry was no different than ancient animal breeding and plant breeding. You could start with a bunch of organisms and just keep on collecting the one that looked better, and by doing this over a long period of time you could hope that you have a better organism.

When one introduced into this the element of recombination, that also was not substantively different, because you could take two organisms, cross them and then keep on selecting their recombinants. In either case the process was random selection. The cleverest thing that you could do was to devise the appropriate selection scheme, the appropriate screening test by which you could pick the organism that you wanted. One didn't really have to know very much about what was going on inside, one could work with a black box approach. This, I am afraid, will not work.

What are these genes doing, how are they expressed, what are the control mechanisms, you have to know all this. So I have no doubt that, as time goes on, the practical problems that one encounters with recombinant DNA technology in genetic engineering will be solved, and people will think of ways for instance of making the Insulin gene work and certainly it will not be a formidable problem if you take a long term view of things, but it is clear that these things cannot be done in a blind and random way. One would have to do this with much more knowledge of the biochemistry of what is going on in the cell. This brings me really to the end of what I have to say. I will try to summarize what I said in two sentences by saying that as far as genetics of antibiotic production goes, there seems to be a large gap between the knowledge that we have, the present knowledge and the knowledge that is possible with the present methods. There also seems to be a gap between knowledge and its application to industrial microbiology. I believe that, in the years to come, this gap will be rapidly reduced. I especially believe this, because in the era of genetic engineering it will be necessary to eliminate this gap before genetic engineering can be successfully applied to microbial industry.

THE β-LACTAM ANTIBIOTICS — PRESENT SITUATION

by

Dr. E. Schrinner

Dr. E. Schrinner studied Veterinary Medicine at the Free University of Berlin from 1952 to 1957 and obtained his Doctorate in Veterinary Medicine.

He worked at the Institute for Animal Health, University of Munich, from 1957 to 1963 and was on temporary secondment as Veterinarian to the Government of Libya in the years 1961 and 1962.

Dr. Schrinner joined Hoechst AG, West Germany, in 1964 and until 1978 worked in the Bacteriology Laboratory of Hoechst AG and was involved in research on Chemotherapy.

Since 1978, Dr. Schrinner is Head of Pharma Research-Chemotherapy, Hoechst AG, Frankfurt, West Germany.

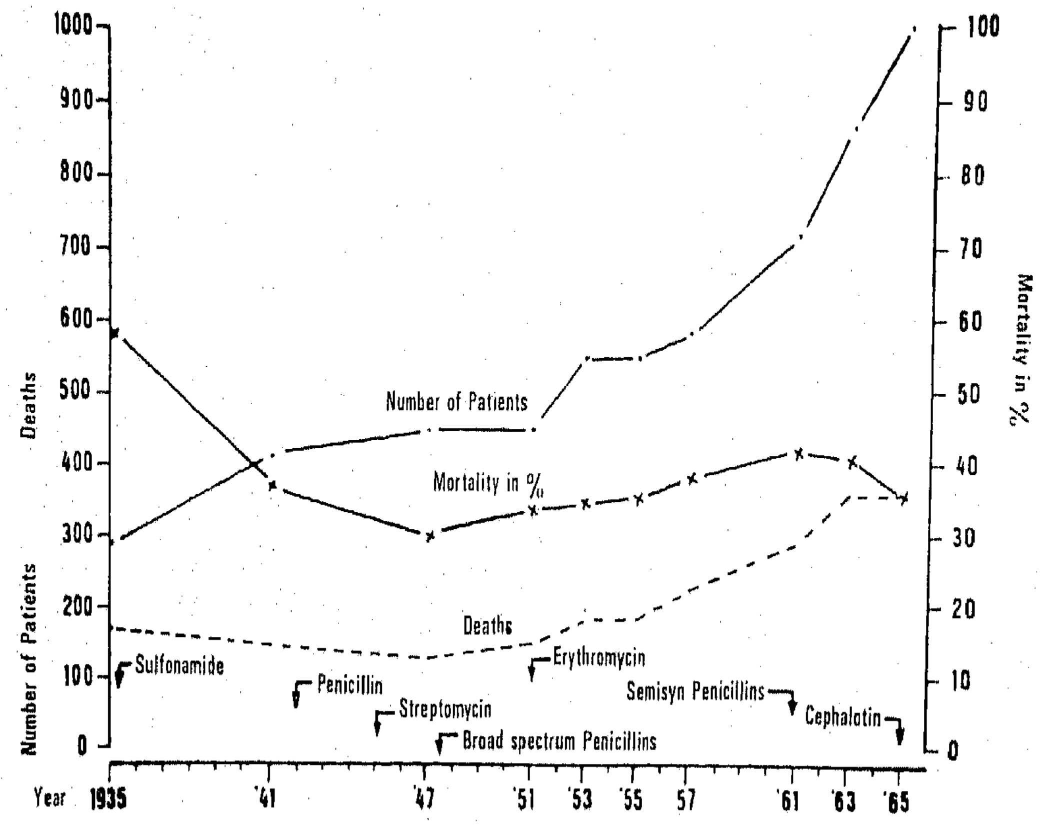
Dr. Schrinner is a member of the American Society of Microbiology, Paul Ehrlich Society for Chemotherapy and the Japanese Society of Chemotherapy.

Ladies and Gentlemen,

As Prof. Zaehner pointed out, last year the 50th anniversary of the penicillin era was celebrated. The penicillins came into use only in the 1940s. But, if we include the sulfonamides, then we have had almost half a century of chemotherapy with very powerful antibacterials.

The first figure (Fig. 1) shows you the statistics which Maxwell Finland compiled for this period, a few years ago, from the Boston Civil Hospital. He clearly showed that in his hospital the rate of mortality decreased rapidly with the introduction of the sulfonamides. But the introduction of other antibiotics did not change the mortality rate any more.

But it would certainly be very wrong to look only into the mortality rate and not look into the age distribution, because very many young people died of acute infection and today we are aware that the people dying of acute infections are becoming less. The chronic infection dominates and people with underlined diseases and with immune deficiencies are the greater proportion of people who are dying today.



by M. Finland

Fig. 1

The second figure (Fig. 2) shows the kind of antibacterials that are available. We have a large range of β-lactam antibiotics. But also we have aminoglycosides, the tetracyclines, the macrolides, the lincomycins, chloramphenicol, sulfonamide now in combination with Trimethoprin and the urinary tract antiseptics. Those areas are shaded where the maximum research has been carried out during the past few years.

The Antibacterials

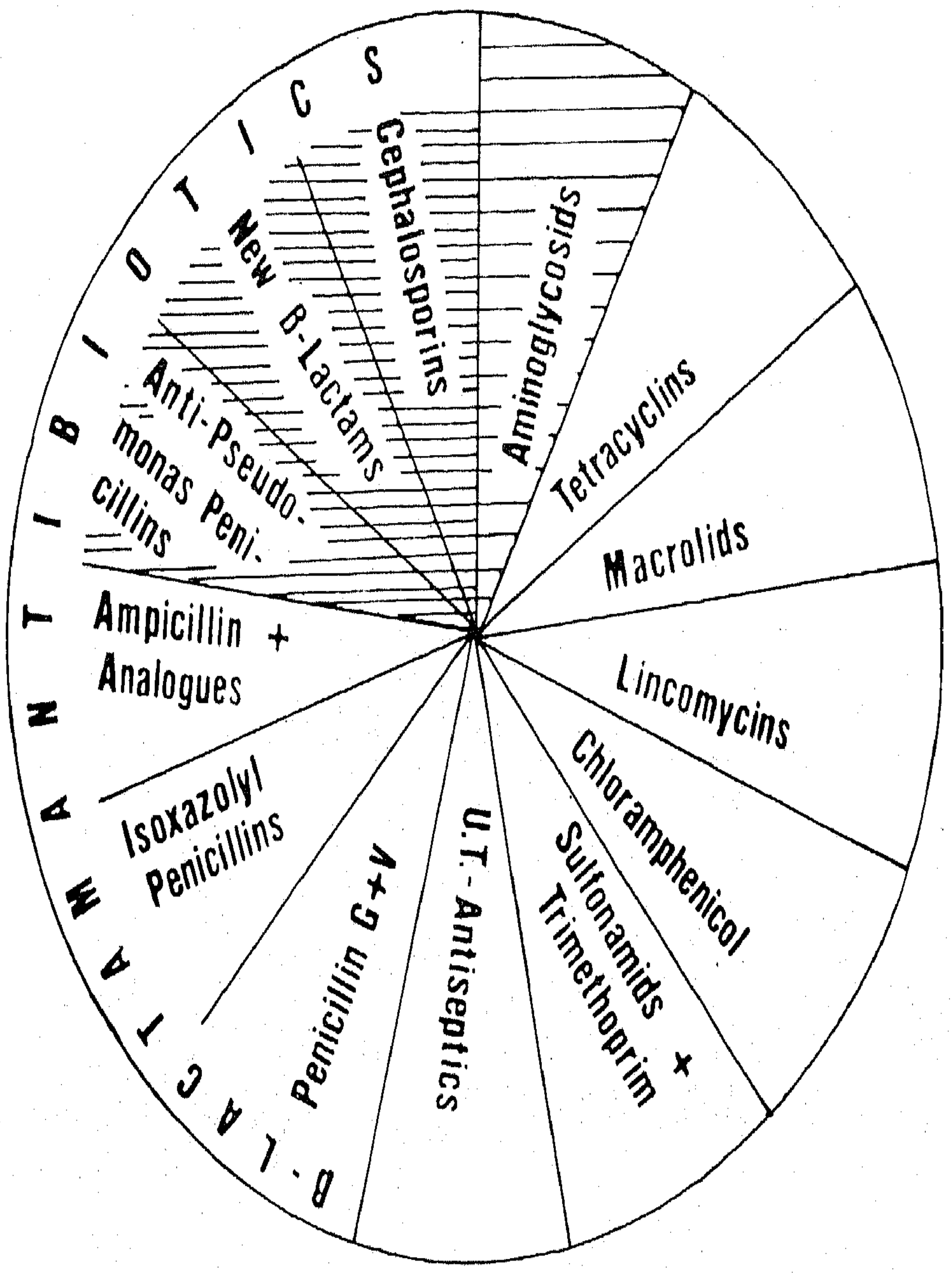


Fig. 2

Figure 3 shows the β-lactams. The areas that are marked indicate the areas of maximum research. Everybody is aware of Penicillin G and Penicillin V the first penicillins. Then came the *Staphylococcus aureus* penicillinase resistant Isoxazolyl penicillin and then the broad spectrum penicillins of the Ampicillin type, the anti-pseudomonas penicillin like Carbenicillin and then the Cephalosporins. The new types of β-lactam antibiotics have also been shown.

ß - Lactamantibiotics

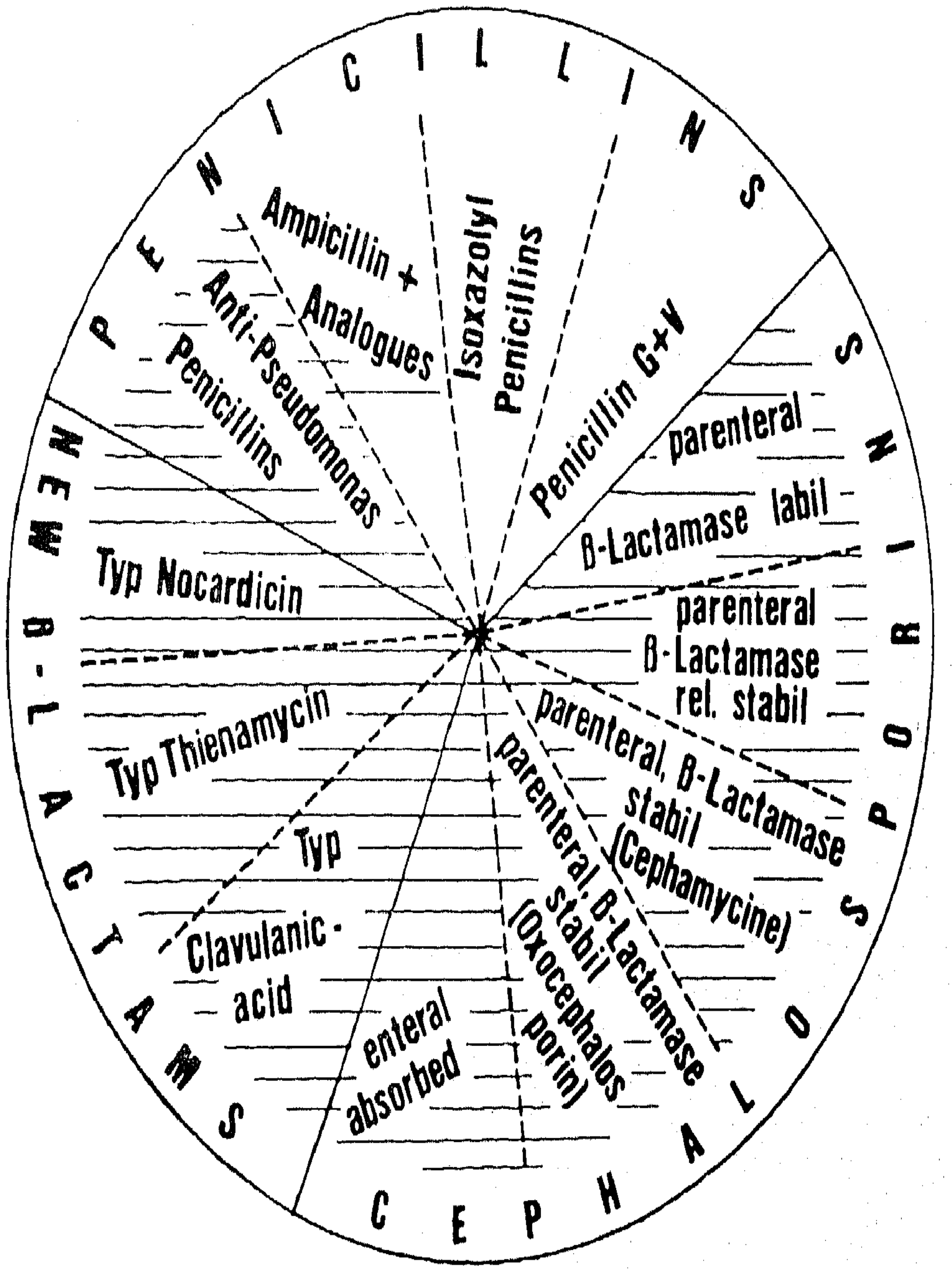

PENICILLINS
CEPHALOSPORINS
NEW ß-LACTAMS
Ampicillin + Analogues
Anti-Pseudomonas Penicillins
Isoxazolyl Penicillins
Penicillin G+V
parenteral ß-Lactamase labil
parenteral ß-Lactamase rel. stabil
parenteral, ß-Lactamase stabil (Cephamycine)
parenteral, ß-Lactamase stabil (Oxocephalosporin)
enteral absorbed
Typ Nocardicin
Typ Thienamycin
Typ Clavulanic-acid

Fig. 3

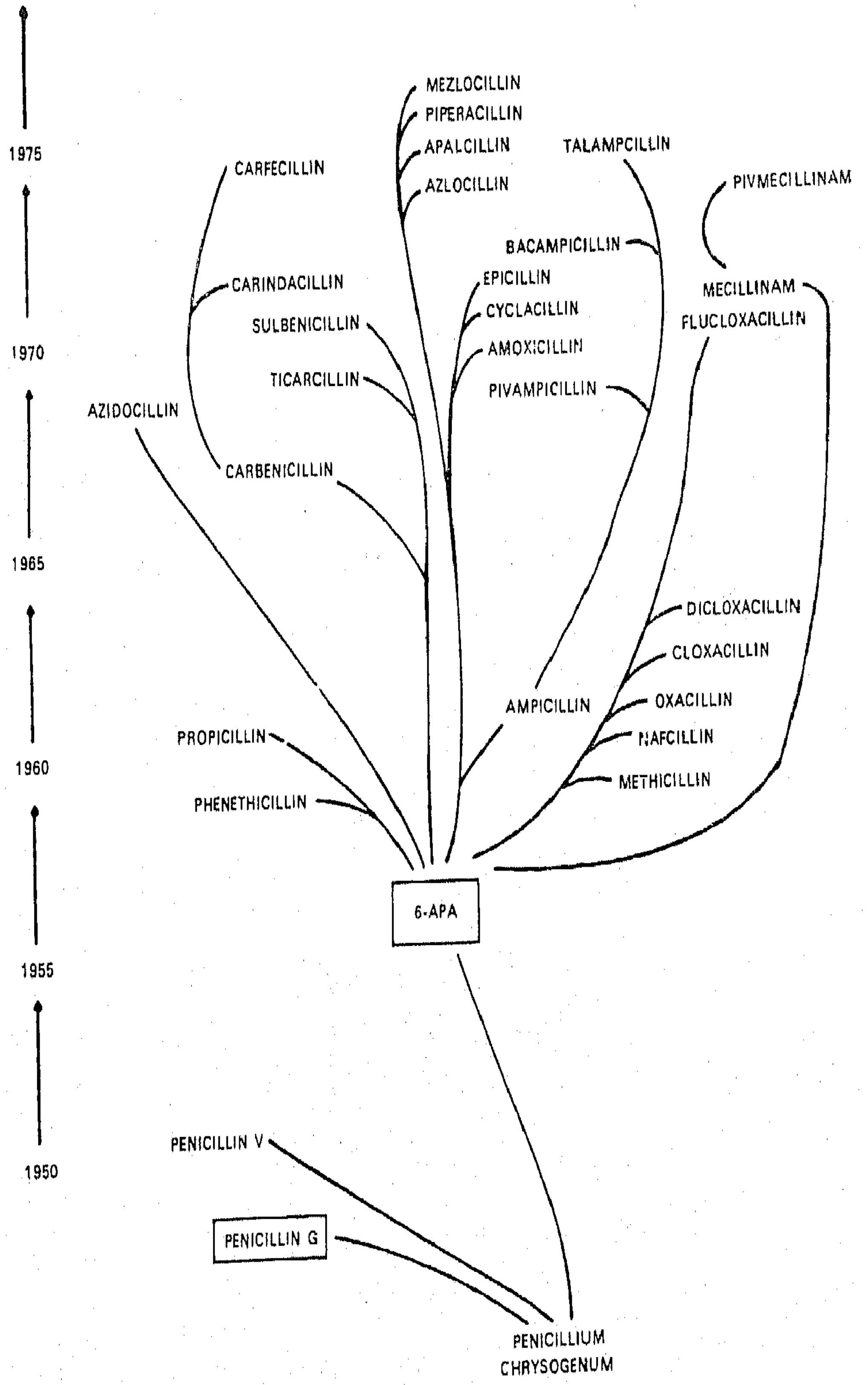

1975
1970
1965
1960
1955
1950
MEZLOCILLIN
PIPERACILLIN
APALCILLIN
AZLOCILLIN
TALAMPCILLIN
CARFECILLIN
PIVMECILLINAM
BACAMPICILLIN
EPICILLIN
CYCLACILLIN
AMOXICILLIN
PIVAMPICILLIN
CARINDACILLIN
SULBENICILLIN
TICARCILLIN
MECILLINAM
FLUCLOXACILLIN
AZIDOCILLIN
CARBENICILLIN
DICLOXACILLIN
CLOXACILLIN
OXACILLIN
NAFCILLIN
METHICILLIN
AMPICILLIN
PROPICILLIN
PHENETHICILLIN
6-APA
PENICILLIN V
PENICILLIN G
PENICILLIUM CHRYSOGENUM

Fig. 4

Figure 4 is a modification of the slide which George Rolinson from Beecham once showed. We have *Penicillium chrysogenum* which is the producing organism of the Penicillins and 6APA. There is one branch leading to Penicillin V, the first orally absorbed compound because of its acid stability. Then we have branches leading to the broad spectrum penicillins, ampicillin derivatives, and the ureido penicillins which are the latest developments. Now you see that the first report of the penicillins was in 1929 and it took up to the 1940s to develop those compounds and still a lot of development is going on around here in the 1970s. And this development still continues.

Figure 5 shows the other great branch of β-lactam antibiotics, namely, the cephalosporins. We have cephalosporium species producing Cephalosporin C and we have streptomyces species producing the Cephamycins, Clavulanic acid and Thienamycin. Going up from Cephalosporin C, we come to 7-ACA and, on the left hand side, I have listed the cephalosporins that are orally absorbed and, on the right hand side, those compounds that have to be injected. Around the 70s you see that a lot of development is going on mainly in the direction of those compounds with greater potency and also resistant to Gram-negative β-lactamases. All the cephalosporins are comparatively more stable than the penicillins against *Staphylococcus aureus* β-lactamase. But only a few of them (the latest developments) are resistant to β-lactamases from Gram-negative organisms. Dr. Coombes has pointed out already that Cefoxitin and Cephamycin derivatives were the first cephalosporins with a 7-methoxy group. The 7-methoxy group stabilises these compounds against almost all β-lactamases. A positive development was Cefmetazole with a higher intrinsic activity than Cefoxitin. And now Cefmetazole is produced from Cephalosporin C. So we have there the linkage between the cephalosporins and the cephamycins.

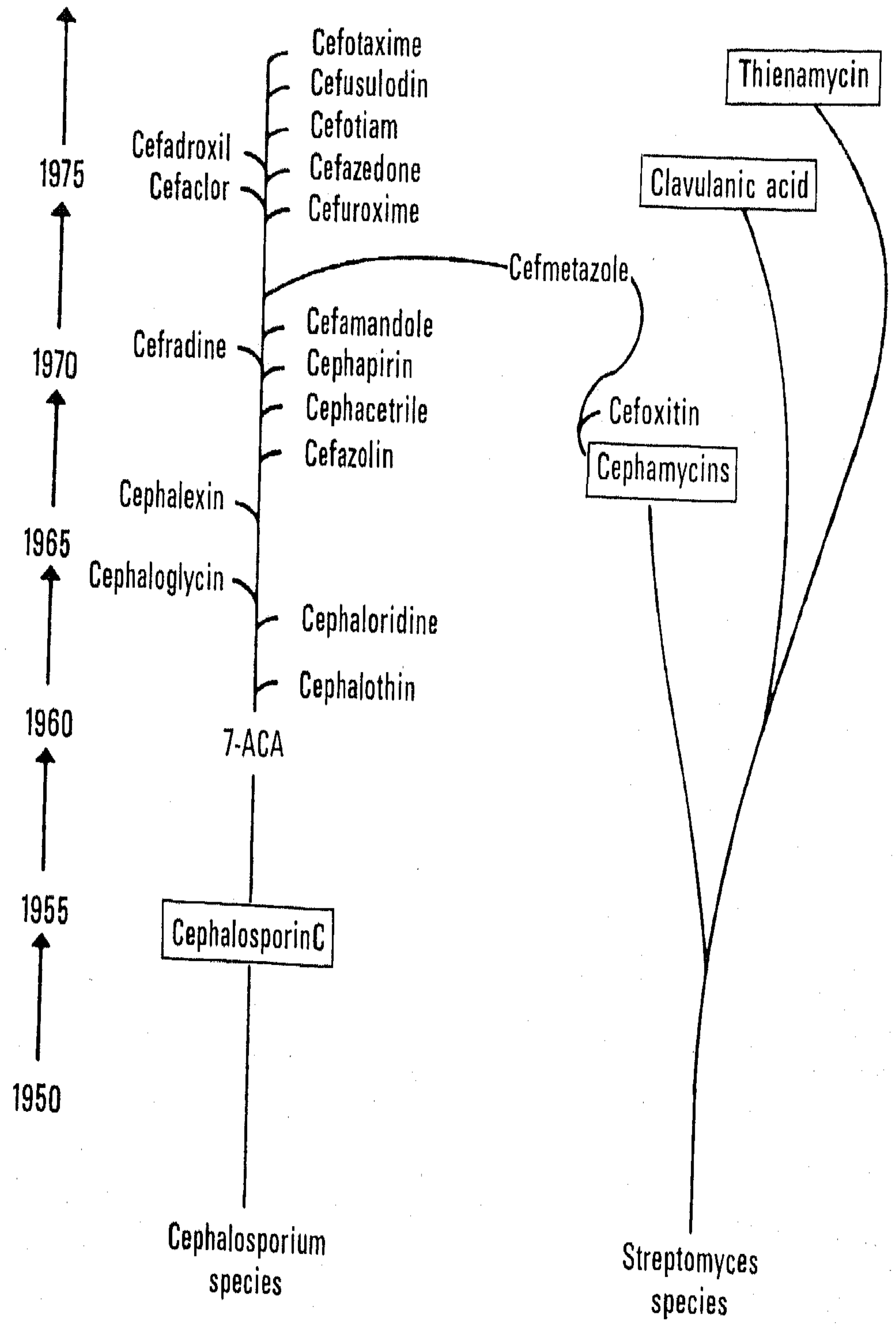

1975
1970
1965
1960
1955
1950
Cefotaxime
Cefusulodin
Cefotiam
Cefazedone
Cefuroxime
Cefadroxil
Cefaclor
Cefmetazole
Cefamandole
Cephapirin
Cephacetrile
Cefazolin
Cefradine
Cephalexin
Cephaloglycin
Cephaloridine
Cephalothin
7-ACA
CephalosporinC
Cephalosporium species
Thienamycin
Clavulanic acid
Cefoxitin
Cephamycins
Streptomyces species

Fig. 5

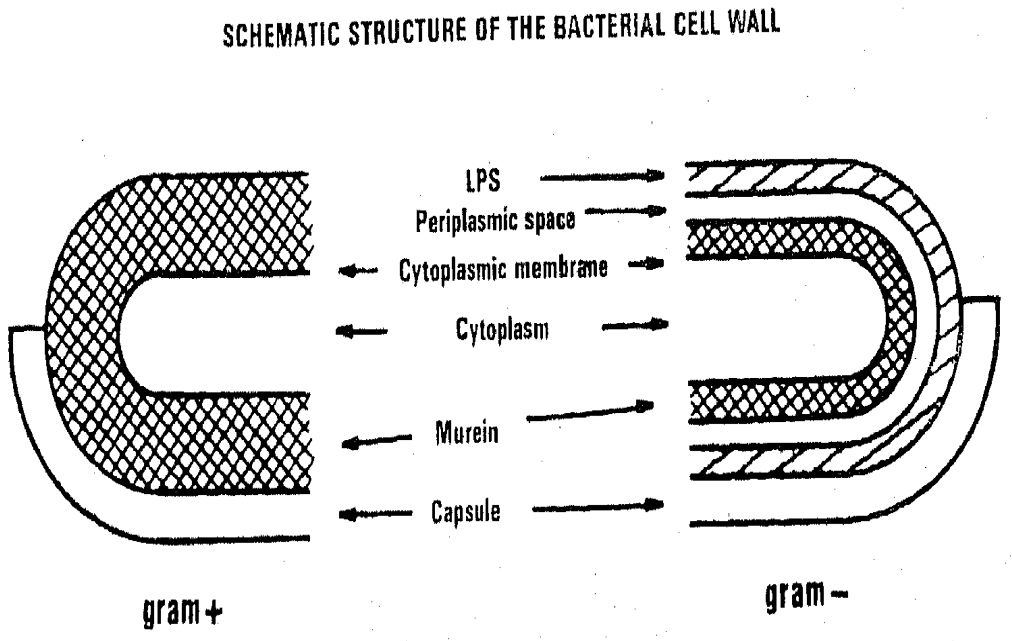

SCHEMATIC STRUCTURE OF THE BACTERIAL CELL WALL
LPS
Periplasmic space
Cytoplasmic membrane
Cytoplasm
Murein
Capsule
gram+
gram−

Fig. 6

The question which interests us is how β-lactam antibiotics work. Figure 6 shows you a section cut through bacteria. We see that Gram-negative bacteria are surrounded by probably a capsule and a lipopolysaccharide membrane. Gram-positive as well as Gram-negative bacteria have a rigid cell-wall layer of murein, and this rigid cell-wall layer is a target for almost all β-lactams antibiotics. But the problem always is that the biosynthesis takes place here in this area of the cytoplasmic membrane. Therefore, all the compounds have to penetrate the cell-wall before they can become active or to come to the active side.

LOCALISATION OF THE β-LACTAMASE

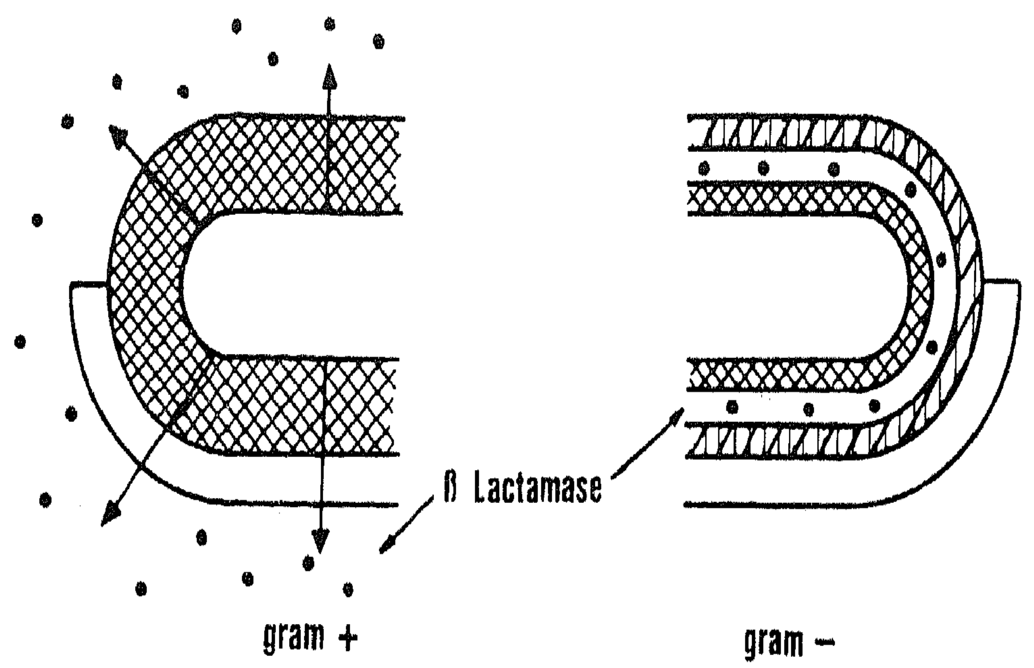


Fig. 7

The Figure 7 shows that on the way to get to the target enzyme there is a possibility, first, to be excluded by the outer cell-wall layer as I have pointed out and the next is that they may be inactivated by the β-lactamases. The Gram-positive organisms produce a large amount of β-lactamase and this is distributed to the surrounding areas. The Gram-negative strains have the β-lactamases concentrated in the periplasmic place. So in order to reach the site of action, the antibiotic has not only to penetrate but also to pass through an area where it might be destroyed by the enzyme and this is a question of speed in passing through this area.

The first way in which a β-lactam compound can be inactivated is by an opening of the β-lactam ring by a β-lactamase (Fig. 8). With the penicillins this will lead to penicilloic acid. Another possibility is that an amidase will split off the side chain and then we get 6-APA. A β-lactam antibiotic with an open β-lactam ring is always antibacterially inactive.

The same is true for the cephalosporins (Fig. 9). Both the β-lactam ring and the side chain can be cleaved. But, in addition, there are esterases which can split off the acetyl group if there is such a substitution in the 3-position. As soon as we exchange with a heterocyclic side chain this possibility is lost.

The cell morphological results with the action of β-lactam antibiotics show that the rigid cell-wall is perforated and we find the formation of protoplasts surrounded only by a membrane and, therefore, they form spheres. They can also form long forms because the dividing septum is lost. But later on a division can take place and we see protoplast formation. In a normal medium, these protoplasts will burst and we get the lysis of the bacterium.

ENZYMATIC DEGRADATION OF PENICILLINS

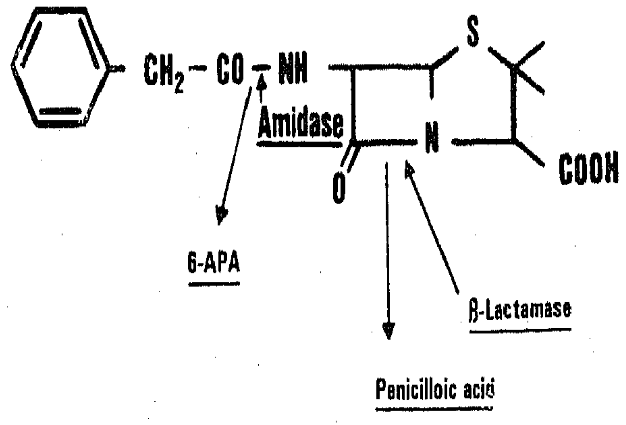


Fig. 8

ENZYMATIC DEGRADATION OF CEPHALOSPORINS

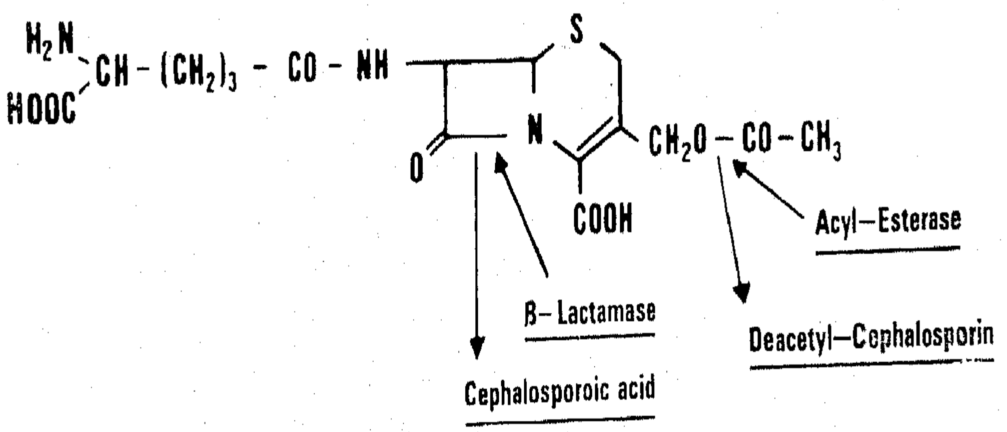
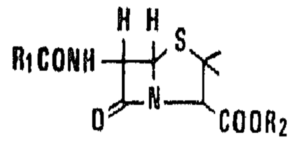


Fig. 9

Now look at the differences in the penicillins. I showed you that the maximum research in the penicillin field at the present time is in the area of the pseudomonas active penicillins. In Figure 10, all those compounds are listed which are on the market or in clinical development. The first one was, of course, Carbenicillin. But, Carbenicillin was not, originally, considered to be active against pseudomonas but considered mainly to be active against indole positive proteus. Later on, it was found that in high concentrations it was active against pseudomonas. We know that the M.I.C.'s of Carbenicllin are around 100 mcg/ml. Therefore, we have to go to very high dosage levels to achieve this concentration in therapy. A dosage as high as 30 gms/day by infusion has to be given. Therefore, if somebody talks of Carbenicillin as a pseudomonas active compound we must take into account this extremely high dosage level. So everybody in the pharmaceutical industry tries to improve this activity. Ticarcillin from Beecham was the next compound where you could see an increased activity. An increased activity of about

Penicillins active against Pseudomonas



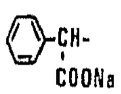
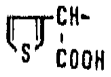
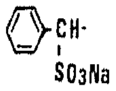
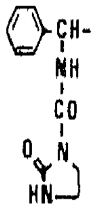
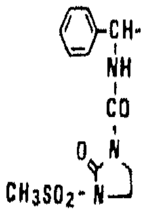
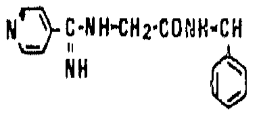
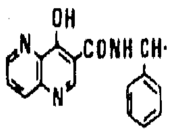
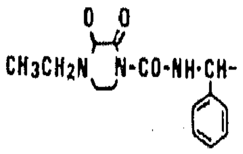
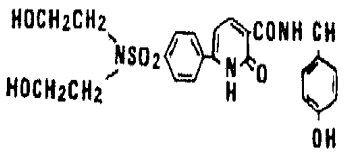
Generic name	Company	R_1	R_2	Appl. Oral	Appl. parenteral
Carbenicillin	Beecham		Na	–	x
Ticarcillin	Beecham		H	–	x
Sulbenicillin	Takeda		H	–	x
Azlocillin	Bayer		H	–	x
Mezlocillin	Bayer		H	–	x
Pirbenicillin	Pfizer		H	–	x
Apalcillin Pc 904	Sumitomo		H	–	x
Piperacillin T 1220	Toyama		H	–	x
CI 867	Warner-Lambert		Na		

Fig. 10

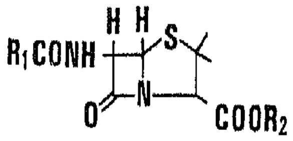
2 to 4 times that of Carbenicillin. Whether this is a real advantage in the clinic always depends on the question whether you can dose it as high as Carbencillin. Sulbenicillin by Takeda, I think, is in the same range and is only used in Japan. Then you have a list of new penicillins called the ureido penicillins, headed by Azlocillin from Bayer. There you have an activity which is a lot better than Carbenicillin but the dosage level is around 16 gms/day. This means you cannot reach the same peak level as with Carbenicillin. Mezlocillin is not active against *Pseudomonas*, but it is listed here because it is an ureido penicillin. Its main activity is against *Klebsiella*. I do not know whether Pirbenicillin is still under clinical trial now because Pfizer has taken over Piperacillin from Toyama on a world wide licence and will probably develop this compound. The other compounds listed are not in the market yet but under clinical trials. Apalcillin from Sumitomo is registered in Japan but not yet in the market. It has an activity against *Pseudomonas* which is in the same range as Azlocillin but not superior to it. The activity of Piperacillin against *Pseudomonas* is not quite as good as Azlocillin but the spectrum is very broad and this includes some of the activities of Mezlocillin too. The latest compound which came into clinical trial is CI 867 from Warner-Lambert. This compound has just entered phase 1 trial in the U.S. As you can see, many companies are trying to improve the activities of compounds in the direction of having better M.I.C.'s against *Pseudomonas*. But nobody really looks only for a pseudomonas compound but also for broad spectrum penicillins.

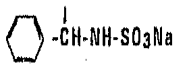
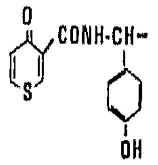
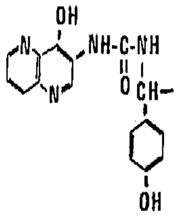
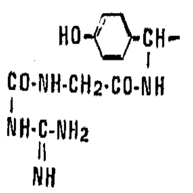
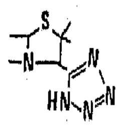
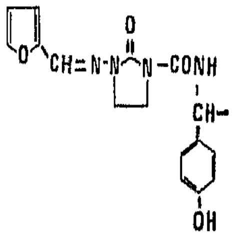
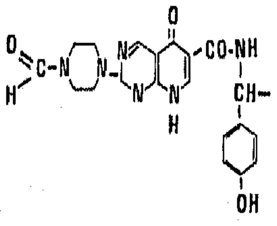
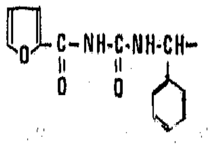
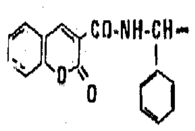
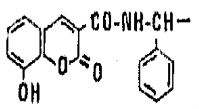
Figure 11 shows you how difficult it is to really improve compounds in this area. These are drop outs from the last few years. Those compounds entered into clinical trials and were then withdrawn because they were not superior to the compounds already in the market. So this demonstrates only that, if you are going into clinical trials, this does not mean that you are also going into the launching and that you will make money out of it. All those compounds cost a lot of money but never made a single rupee.

One disadvantage of all those compounds shown is that they are only parenteral compounds. They have to be administered by injections or infusion They are not enterally absorbed and, therefore, cannot be given orally. One way of achieving enteral absorption is by esterification. Carindacillin and Carfecillin are two examples of esterified compounds with better enteral absorption (Fig. 12). But, in order to treat a pseudomonas infection, you will need a high concentration of Carbenicillin. Therefore, if you want to use an orally absorbed ester you would have to administer upto 30 gms and that is impossible because of gastrointestinal side-effects. Therefore, many people are against the use of these compounds because you are selecting resistance against Carbenicillin by underdosing. The only use of these compounds is in urinary tract infections where a concentration is reached later on in the urine, and even this is not without question because you do not know whether the infection is localised or is spread in the surrounding tissues.

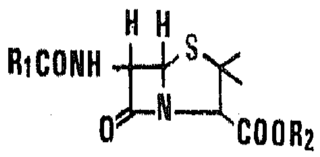
Fig. 11

Penicillins active against Pseudomonas



Generic name	Company	R_1	R_2	Appl.	
				Oral	Parenteral
Suncillin	Bristol		H	—	X
Timoxacillin Pc 455	Yamanouchi		H	—	X
EMO 32 412	Merck		Na	—	X
CP 38 118	Pfizer			—	X
Furazlocillin Bay K 4999	Bayer		Na	—	X
PL 385	Dainippon		Na		
Furbenicillin BL-P 1654	Bristol		K	—	X
TEI-1194	Teijin Inst. Tokyo		H		
TEI-2012	Teijin Inst. Tokyo		H		

Penicillins active against Pseudomonas



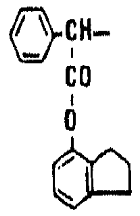
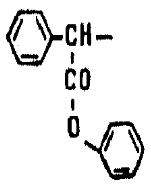
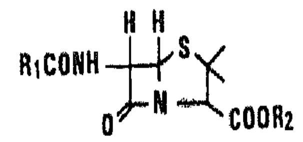
Generic name	Company	R_1	R_2	Appl.	
				Oral	parenteral
Carindacillin	Pfizer		Na	X	—
Carfecillin	Beecham		Na	X	—

Fig. 12

We come to the Ampicillin analogs (Fig. 13). Starting with Ampicillin as the first broad spectrum penicillin with activity also against enterobacteriaceae and which is orally absorbed. The absorption rate of Ampicillin is 40-60% depending on the individual. There are 2 directions for improvement, one is to increase the antibacterial activity and the other is to increase the absorption rate. Amoxicillin from Beecham shows a slight increase in activity but also has an increased absorption rate. It is very astonishing that only one hydroxyl group, which is the only difference from Ampicillin, increases the absorption by 20% and it is also said that the bacteriolytic effect of this compound is higher than that of Ampicillin. Epicillin from Squibb shows almost no difference in activity and pharmacokinetics. Azidocillin from Beecham is a broad spectrum compound but it is not too different from Ampicillin. The same is true for Cyclocillin from Wyeth. Hetacillin forms Ampicillin in the body after being metabolised. A compound with a rather different structure and activity is Mecillinam from Leo. This compound attracted the attention of many people, especially theoretical scientists, because it has a different mode of action. The difference is in its binding to the penicillin binding proteins. It binds to penicillin binding protein 2 while other β lactams bind to other sites. From its biological activity spectrum you see that it is not very active against Gram positive organisms but is active against a broad range of enterobacteriaceae. Esters of Ampicillin and esters of Mecillinam show again an improved absorption rate. The increase in absorption is also around 20% and this means that they are not better than that of Amoxicillin. In addition to this, it is

Penicillins of the Ampicillin Type



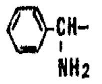
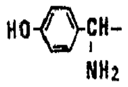
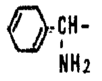
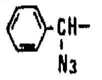
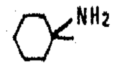
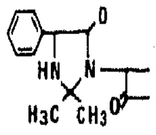
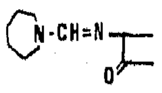
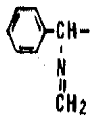
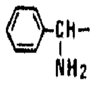
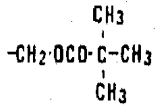
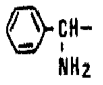
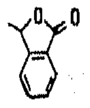
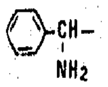
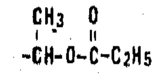
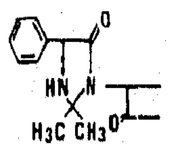
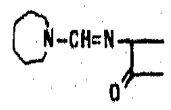
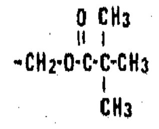
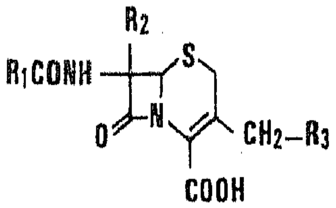
Generic name	Company	R_1	R_2	Appl.	
				Oral	parenteral
Ampicillin	Bristol		H	×	×
Amoxycillin	Beecham		H	×	×
Epicillin	Squibb		H	×	×
Azidocillin	Beecham		H	×	×
Cyclacillin	Wyeth		H	×	×
Metacillin	Bristol		H	×	×
Mecillinam	Leo		H	–	×
Methampicillin	Midy		Ca	×	×
Pivampicillin	Leo MSD		 $-CH_2{\cdot}OCO{\cdot}C(CH_3)_2{-}CH_3$	×	–
Talampicillin	Yamanouchi Beecham			×	–
Bacampicillin	Asta		 $-CH(CH_3){-}O{-}C(=O){-}C_2H_5$	×	–
Sarpicillin	Bristol		$-CH_2OCH_3$	×	–
Pivmecillinam	Leo		 $-CH_2{-}O{-}C(=O){-}C(CH_3)_2{-}CH_3$	×	–

Fig. 13

possible that some esterases could split off the ester group. This might also lead to liver toxicity. But now people are convinced that there is no liver toxicity. But any-how the individual variation in absorption rate does not allow them to be dosed lower than Ampicillin.

Coming to the Cephalosporins (Fig. 14) we can start with Cephalothin and Cephaloridine, and the question is in what direction shall we improve these compounds. Cephaloridine is reported to have nephrotoxicity and Cephalothin to have some local irritation at the site of injection, and certainly we should increase the spectrum. Since the half lives are very short, it would be an improvement if we could increase them so that we can dose less frequently. When Cefazolin came from Fujisawa we found out that it was already an improvement because the half life time was prolonged to almost 2 hours and the compound also had an increased activity compared to Cephalothin. It was a question whether this change in half life time, this change in pharmacokinetics, is only due to its high protein binding. (85% to serum albumin). Therefore, one has to ask whether the distribution in the body is equal and whether this is not a kind of cosmetic of the pharmacokinetic. I really don't want to go into this question whether the protein binding means something in relation to its activity, because if you take Ceftazole, for instance, this is just missing a methyl group from Cephazolin. This compound is bound around 25-30% to serum albumin. The half life time is reduced almost to half but its activity in clinical practice is just as good even if you administer in the same sequence as you administer Cefazolin. The in vitro activity of both compounds is comparable. Cefacetrile is not too different from Cephalothin. Cephapirin and Cefazoflur show some increase in activity but, if you look further down, you will see that most of these compounds are not in the market but only in clinical trials. Only Cefazedone is marketed in Germany by Merck. All these compounds show broad spectrum activity compared to Cephalothin and the activity increases as you go down the list. Most of them are stable to β-lactamases from *Staph. aureus* but susceptible β -lactamases from Gram negative bacteria.

CEPHALOSPORINS



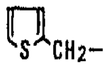
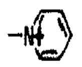
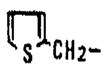
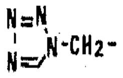
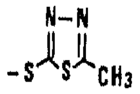
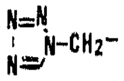
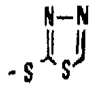
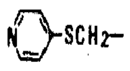
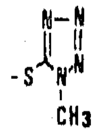
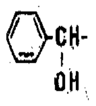
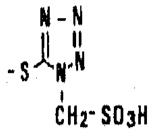
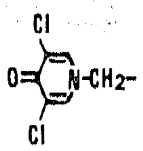
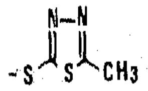
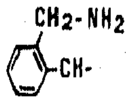
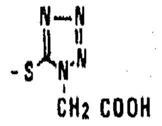
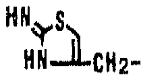
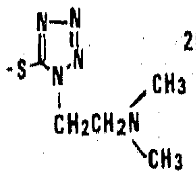
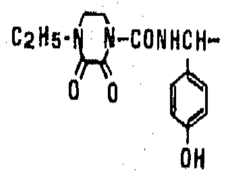
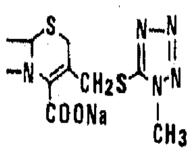
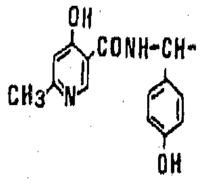
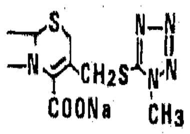
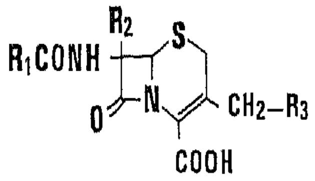
Generic Name	Company	R1	R2	R3	Appl. Oral	Appl. Parenteral	150 i. v.
Cephalordine	Lilly, Glaxo		H		-	x	90′
Cephalotin	Lilly, Glaxo		H	$-OCOCH_3$	-	x	30′—40′
Cefazolin	Fujisawa		H		-	x	105′
Ceftezole	Chugai Fujisawa		H		-	x	68′
Cephacetrile	Ciba	$NC-CH_2-$	H	$-OCOCH_3$	-	x	40′—80′
Cephapirin	Bristol		H	$-OCOCH_3$	-	x	24′—48′
Cefazaflur	SK & F	$CF_3 - SCH_2-$	H		-	x	43′
Cefonicid SK & F 75 073	SK & F		H		-	x	240′
Cefazedone	Merck		H		-	x	98′
Ceforanid BL-S 786	Bristol		H		-	x	180′
Cefotiam SCE 963 CGP 14 221E	Takeda		H	 2HCl	-	x	
Cefoperazone T 1551	Toyama		H		-	x	125′
SM 1652	Sumitomo		H		-	x	

Fig. 14

CEPHALOSPORINS



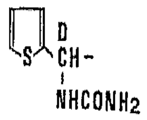
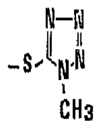
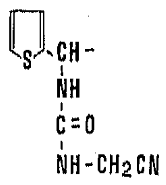
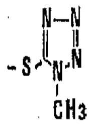
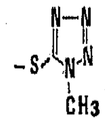
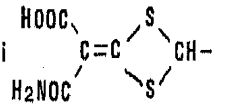
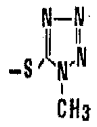
Generic name	Company	R1	R2	R3	Appl. oral	Appl. parenteral	150 i.v.
SQ 14,359	Squibb/ Heyden		$-OCH_3$		—	X	
SQ 14,448	Squibb/ Heyden		$-OCH_3$		—	X	
SK & F 73 678	SK & F	CF_3SCH_2-	$-OCH_3$		—	X	
YM 09330	Yamanouchi		$-OCH_3$				

Fig. 15

Figure 15 shows the dropouts during the last few years from the Cephalosporin group of compounds.

In order to broaden the spectrum or to increase the stability against β-lactamases, there are two approaches. One of them produced Cefuroxime (Fig. 16). With this steric conformation the β-lactam ring seems to be stabilised. Cefuroxime is stable to most of the gram negative β-lactamases. The stability is not absolute but it is high enough so that the compound is active against those organisms which produce these β-lactamases.

Cefotaxime shows the same type of stability to β-lactamases as Cefuroxime but, in addition, it shows an increased activity against many Gram negative bacteria. Against Gram positive bacteria, the activity is similar to that of the other Cephalosporins. But against Gram negative bacteria the activity is one or two or even three logs better. It is highly active against species of *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter* but not against anaerobes such as *B. fragilis*. The

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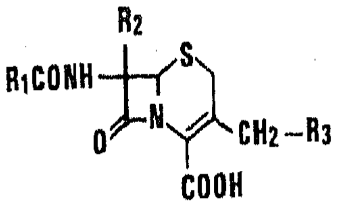


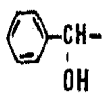
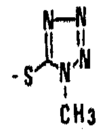
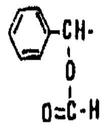
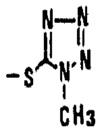
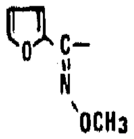
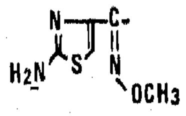
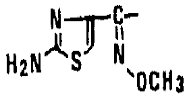
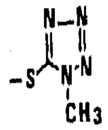
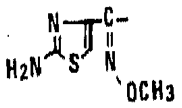
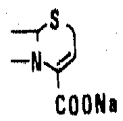
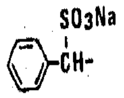
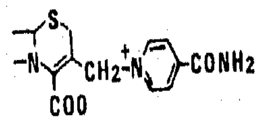
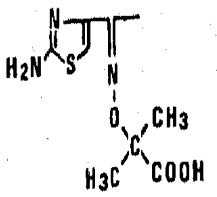
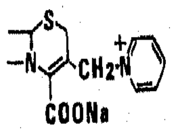
Generic name	Company	R_1	R_2	R_3	APPl.		
					Oral	Parenteral	150 i.v.
Cefamandole	Lilly		H		-	×	34'
Cefamandole Nafate	Lilly		H		-	×	34'
Cefuroxime	Glaxo		H	$-OCONH_2$	-	×	65'–70'
Cefotaxime HR 756	Hoechst-Roussel		H	$-OCOCH_3$	-	×	75'
SCE 1365 RU 24550	Takeda Roussel		H		-	×	
Ceftizoxime FK 749	Fujisawa		H		-	×	80'
Cefsulodin CGP 7174E SCE 129	Ciba Geigy Takeda		H		-	×	84'
GR 20263	Glaxo		H		-	×	96'

Fig. 16

β-lactamase from *B. fragilis* destroys both Cefuroxime and Cefotaxime. Compound SCE 1365 is a development of Takeda. It is in phase I trials in Japan. The only difference is the change in substitution in the 3-position. Cefsulodin from Ciba-Geigy and Takeda is the only narrow spectrum Cephalosporin that may be marketed. It has an increased activity against *Pseudomonas aeruginosa* (1-10 mcg/ml). It has some activity against *Staph. aureus* but that is all. Glaxo has recently described a compound GR 20263. This is a little better than Cefotaxime in its activity against *Pseudomonas*. It has decreased activity against *Staph. aureus*. Cefotaxime was the first compound which showed activity against pseudomonas species but the activity varies from strain to strain as with all these compounds, because none of the compounds cover all the strains.

Another approach to stabilising the β-lactam ring is the introduction of the 7-methoxy group (Fig. 17). Cefoxitin was the first Cephamycin compound to be marketed. It is highly active against a broad range of bacteria but much less active compared to Cefuroxime and Cefotaxime. But Cefmetazole from Sankyo shows a better activity than Cefoxitin and still has the same stability to β-lactamase. Both compounds can also claim to be stable to β-lactamases even from *Bacteroides fragilis*.

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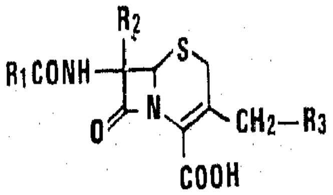


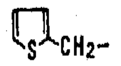
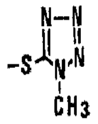
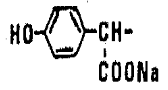
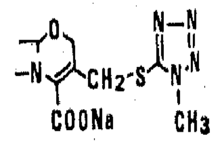
Generic name	Company	R_1	R_2	R_3	APPl.		
					Oral	Parenteral	150 i.v.
Cefoxitin	Merck		$-OCH_3$	$-OCONH_2$	-	×	45'
Cefmetazole CS-1170	Sankyo	$NC-CH_2-S-CH_2-$	$-OCH_3$		-	×	60'
6059-S Lilly 127935	Shionogi Lilly		$-OCH_3$		-	×	108'

Fig. 17

Dr. Coombes has talked of the oxa-cephalosporins. The one from Shionogi which is under trial is the first one in the clinic. In the cephem area, this group increases the stability of the ring, but decreases the penetration through the cell envelope. This group also increases the activity. All three compounds are stable to the β-lactamases known today.

Because of the difficulty in introducing the 7-methoxy group, only two of the compounds shown in Figure 18 were used and have disappeared again. The price of these compounds is prohibitive most probably.

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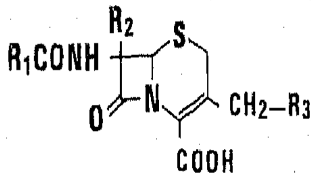


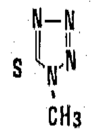
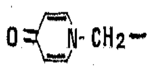
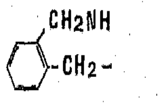
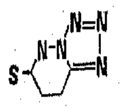
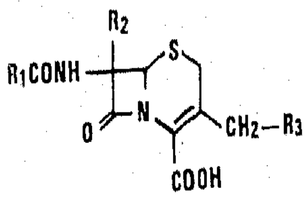
Generic name	Company	R1	R2	R3	Appl. oral	parenteral	150 i.v.
SQ 67 590	Squibb/ Heyden	$NC-CH_2-S-CH_2-$	H			x	40'
RMI 15 307 ZA	Merrell National		H	$-OCOCH_3$		x	
BB-S 226	Bristol-Banyu		H			x	

Fig. 18

Next we come to the orally absorbed Cephalosporins (Fig. 19). The glycine side chain is necessary for the absorption. Cephalexin is almost completely absorbed. But the activity is rather low. Cephaloglycin has better activity but absorption is only 20-25%. Cephradine from Squibb has one double bond less but has the same activity as Cephalexin. Cefatrizine has higher activity and Cefaclor (from Lilly) has 2-8 times better activity then Cephalexin. But the stability is lower. Blood levels are not so high. It is not as resistant to β-lactamases. But it has good activity against *H. influenzae*. CGP 9000 from Ciba-Geigy is under trial and Cefadroxil

CEPHALOSPORINS



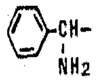
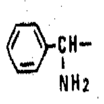
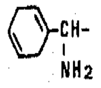
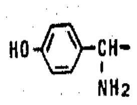
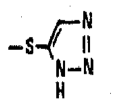
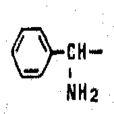
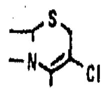
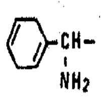
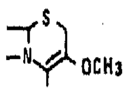
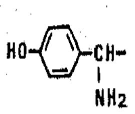
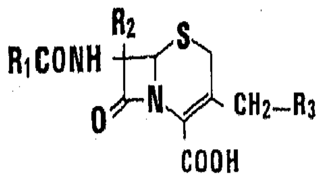
Generic name	Company	R1	R2	R3	APPl. Oral	Parenteral	150 i.v.
Cephalexin	Lilly Glaxo		H	H	X	–	40'-90' p.o.
Cephaloglycine	Lilly		H	$-OCOCH_3$	X	–	60' p.o.
Cephradine	Squibb		H	H	X	X	40' i.v. 45' p.o.
Cefatrizine	Bristol-Myers SK & F		H		X	X	60'
Cefaclor	Lilly		H		X	X	40'-45'
CG-P 9000	Ciba-Geigy		H		X	–	36'-45' p.o
Cefadroxil	Bristol		H	H	X	–	70'-90' p.o.

Fig. 19

from Bristol also. The final slide shows compounds which have not shown better activities and have, therefore, been dropped. (Fig. 20)

CEPHALOSPORINS



Generic name	Company	R1	R2	R3	Appl.		
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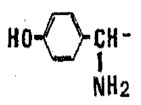
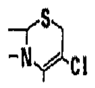
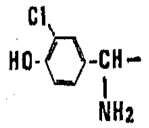
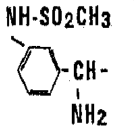
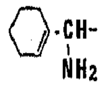
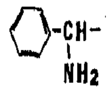
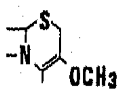
Lilly 98 249	Lilly		H		X		
BLS-547	Bristol-Myers		H	H			
Cefsumide FR 10612	Fuji sawa		H	H	X	—	70' p.o.
SCE 100	Takeda		H	H	X	—	
Lilly 109294 CG-P 3940	Lilly Ciba-Geigy		H		X	—	70'-90' p.o.

Fig. 20

THE β-LACTAM ANTIBIOTICS—FUTURE OUTLOOK

by

Prof. M. Richmond

Professor Mark Richmond studied in Clare College, University of Cambridge, obtaining a Ph.D. in Microbial Biochemistry and an Sc.D. at Cambridge in 1973.

Prof. Richmond worked from 1958 to 1965 at the National Institute of Medical Research. In 1965 he switched over to the University of Edinburgh, where he held the post of Reader in Molecular Biology.

From 1968 until the present day, Prof. Richmond occupies the position of Professor and Head of Bacteriology at the University of Bristol.

Mr. Chairman, Ladies and Gentlemen,

What I am going to say this afternoon will overlap a bit with what Dr. Coombes has said this morning and also Dr. Schrinner. This is really because I think it is quite useful to look a little bit at the past history when we are beginning to think of the future. We start from the point that, in this field that we are looking at, most of the compounds that have been in effective clinical use, in the last few years, have belonged to one of two major groups, namely, the penicillins or penams and the cephalosporins or cephems. The first figure shows the structure of these two basic nuclei.

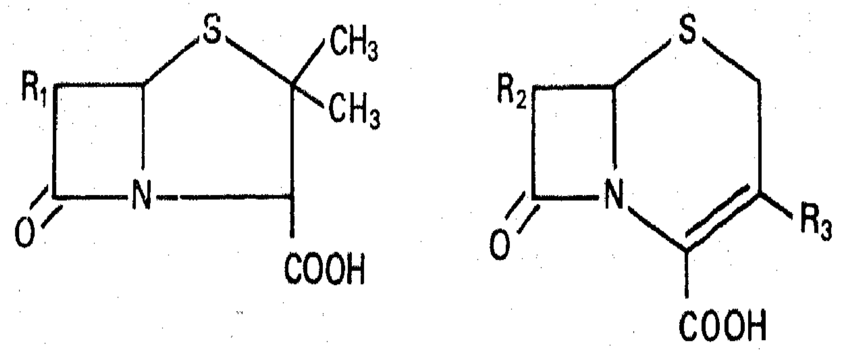


Fig. 1

Now the history of the development of β-lactam antibiotics as therapeutic agents lies with the penicillin series. And the cephalosporins came into the picture only relatively late. Initially, there was developed the entirely natural derivatives of penicillin, the so-called benzyl penicillins or Penicillin G. This is a compound which is active only against the Gram-positives and has very little activity against the Gram-negatives and, as you all know, it is susceptible to the penicillinase of *Staphylococcus aureus* in particular.

Nevertheless, I think it is very important to stress that very large amounts of benzyl penicillin, an antibiotic that has been present for almost 30 years, are still sold and is providing useful treatment for very many people. The first step that was taken from benzyl penicillin was the introduction of the oxygen atom in the side chain which rendered the molecule resistant to acid hydrolysis. Therefore, this derivative, Penicillin V, also took a substantial place in the market because of its possibility of oral administration. The first step that was taken then is a step which is one which has lived through the whole history of β-lactam antibiotics. In other words, as the story has developed, there has always been the need to take this step to develop an oral derivative. And, as you shall see, it is a step which, in certain respects and in certain cases, is difficult to carry out. These two molecules, Penicillin G and Penicillin V, were very active against Gram-positive organisms, notably *Staph. aureus*. They did not have much activity against Gram-negative organisms and the next major advance was the introduction, by the Beecham group, of Ampicillin, where the amino group was inserted in the side chain. Ampicillin is an oral molecule which has a broad spectrum of activity and which, in many respects, is very active, very effective and still very, very widely used. And, finally, in the initial programme of this work, although it took almost 15 years to achieve, there was the first compound with a carboxyl group in the side chain and this was Carbenicillin.

In many ways, in this series of compounds, one has, as it were, the important steps that have been taken in β-lactam research and in many respects all things that have followed have either been attempts to retrace these steps because the utility of these compounds has been lost or there have been attempts to improve on the properties of these types of molecules. It is extremely important to stress a question which is often asked, and which is a very important question, asked usually by Governments, and not so much by the pharmaceutical industry, and that is, why do we need any molecules which are improvements on these four basic types of β-lactam antibiotics? They are, after all, cheap, they are in many respects effective, they are still used in large amounts and, therefore, why do we need new molecules? And it is mainly on this question of why we do need advances on these molecules that I want to talk about. But, I also want to stress, as I go on, where it is that our search for improvement on these molecules are running us into a position where the improvements are getting so expensive that I think it is becoming dubious as to whether they will have a life which will last very long. Now, the first problem that beset the β-lactams when they were introduced into therapeutic practice, was the emergence of resistant bacteria. It was seen first in the Gram-positive organisms. And the next figure (Figure 2) summarises data from the World Health Organisation.

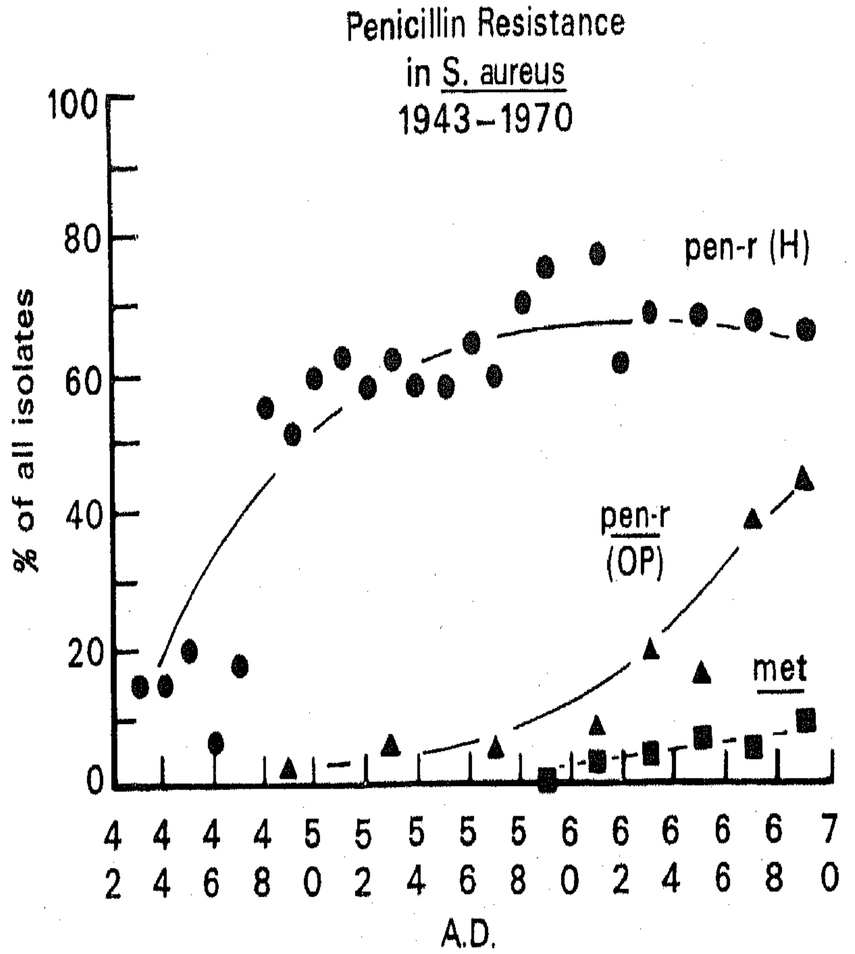


Fig. 2

It shows the incidence of penicillin resistant *Staph. aureus*, in hospitals, for the period 1942, when Penicillin G was first introduced in clinical practice, to 1970. You will see that even when benzyl penicillin was first introduced into hospitals, there were likely to be, we now know that there were, rare penicillinase producing organisms. And very soon after the drug was used therapeutically for the first time, there was an explosion in the occurrence of resistant strains and by the time we come to the middle 1950's, benzyl penicillin and phenoxymethyl penicillin were so blunted, as far as the treatment of *Staph. aureus* infection was concerned, that the clinicians were almost back to the situation of the pre-penicillin era. It is important to stress that this was a response of *Staph. aureus* only. The group A Streptococcus has remained just about as sensitive to Penicillin G and Penicillin V as it was in the beginning; and that is something which is often overlooked by people who are always talking about resistance. There are classes of microorganisms which have not achieved prominence in this way. In Britain, at any rate, the incidence of penicillinase production (penicillin resistance) in *Staph. aureus* outside hospitals was much lower to rise than the incidence in hospitals. And it was only towards the end of the 60's that the incidence of penicillinase production in normal *Staph. aureus* outside hospitals rose to the point where it was the same or similar to that seen inside the hospitals. As I said, towards the end of the 1950's, benzyl penicillin and Penicillin V were so blunted from the point of view of therapy, that a real advance was needed, a molecule was needed which was not hydrolysed by the enzymes. The advance came from Beecham with the discovery of Methicillin, a derivative of benzyl penicillin which is insensitive to hydrolysis by *Staph. aureus* β-lactamase. So we have seen now the first step away from the basic 4 molecules. The need to develop a derivative which remained active, despite the emergence of resistance, and this step, the development of molecules which are active against resistant strains, seems to be a continual problem as far as β-lactam antibiotics are concerned. Because, in general, as soon as a new derivative is introduced one tends to find beginning to appear variants of the microorganisms resistant to the new derivative. So one is always in a process of a continuous battle, as it were, between the innovative powers of the pharmaceutical industry to design newer and more effective compounds and the genetic flexibility and diversity of the microorganism, the evolutionary potential, which all the time leads to organisms capable of resisting antibacterial attack.

With Methicillin, rather soon after it was introduced into clinical practice, around the 60's, resistant strains of *staphylococci* started to emerge; and that process went on but they have not reached anything like the proportion, in most cases, of the total bacterial population as has been the case for other penicillins. Now the problem of Staphylococcus and of Penicillin G and Penicillin V has been repeated with Ampicillin. Ampicillin was an antibiotic that was devised for activity against Gram-negative organisms and the penicillinases of Gram-negative organisms have become much more prevalent. And, as a result, the chances of treating Gram negative infections in hospitals with Ampicillin have correspondingly been reduced. With the emergence of resistance to Penicillin G and Penicillin V, the Cephalosporins entered the field. And the particular reason that they were valuable was that they proved rather rapidly that they were in general β-lactams that were more resistant to hydrolysis by staphylococcal β-lactamases than the penicillins. Therefore, the derivatives of Cephalosporin C, notably Cephalothin and Cephaloridine, found a place quite quickly in the armament of therapy because they could be used as an alternative to Methicillin for the treatment of resistant staphylococcal infections. In practice, the emergence of the cephalosporins as therapeutic entities has become extremely important because one of the ironies of the developments in the β-lactam field has been that, in general, as history unfolds, we see the cephalosporins probably as a more potential source of effective β-lactam antibiotics than the penicillins. So the molecule which arrived on the scene later has turned out ideally to have greater potential and as we shall see later it has come to a point where it has many properties that are better than the properties of the penicillins.

Now, the question of resistance to staphylococcus β-lactamase can be said to have been solved by the introduction of Methicillin, Cloxacillin, Dicloxacillin, Fluocloxacillin, etc., all minor variants. It was also solved for *Staphylococci* by the introduction of the cephalosporins. But the resistance problems posed by the Gram-negative organisms such as *E. coli*, *Pseudomonas*, *Klebsiella*, *Proteus*, *Enterobacter*, etc. was a very much more serious and dominant challenge because it was proved that there was more than one kind of β-lactamase which was causing resistance. Therefore, one has a much bigger task in devising β-lactamase resistant compounds that can be used against the Gram-negatives. One has a much wider range of different penicillinase and cephalosporinase enzymes to deal with. And it is really at this point that the penicillins have fallen down. No one has yet been able to design the penam derivatives which have the breadth of insensitivity to β-lactamases which is necessary for them to be really therapeutically effective in the hospitals. The whole development in the future has been left with the cephalosporins. Now in cephalosporin C, it has been possible to design derivatives which are insensitive to hydrolysis by almost all types of β-lactamases. The surest way to do this is to introduce a methoxy group in the 7-alpha position of the cephem nucleus (Figure 4).

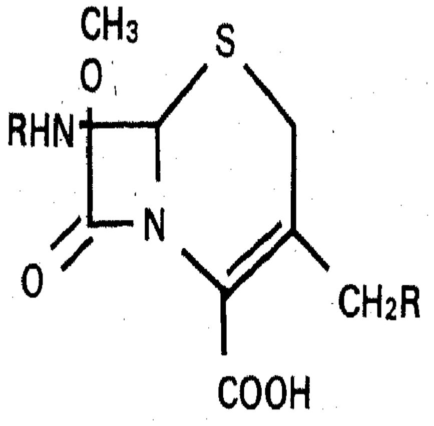


Fig. 3

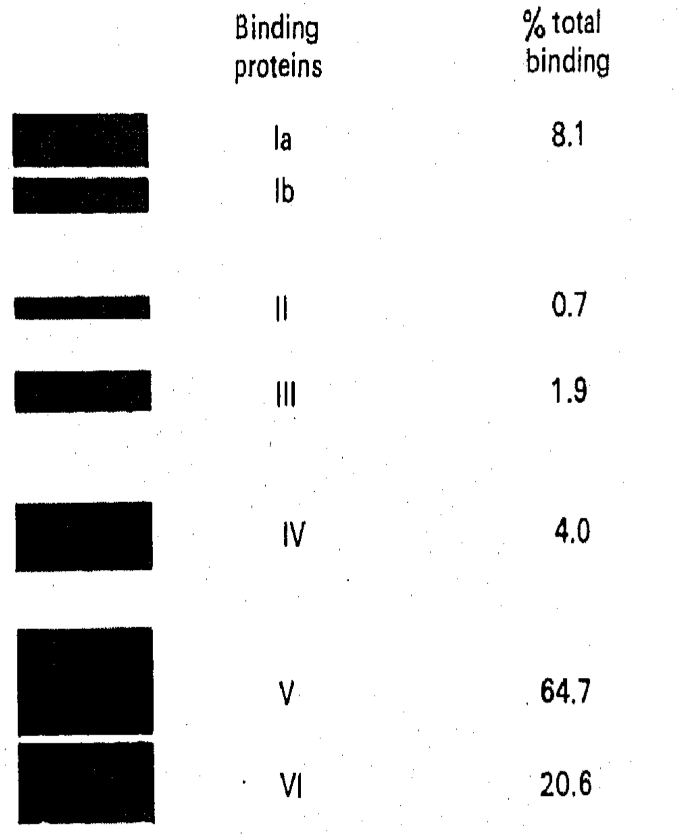
Therefore, one can say, without exception, that cephems with a 7-alpha methoxy substituent are universally resistant to hydrolysis by β-lactamases. So if one wants to fire-proof a β-lactam against β-lactamase attack, one has to insert the 7-methoxy in that position. The trouble is that when you do it, in the very, very great majority of cases, you end up with a molecule that is much less active than the corresponding molecule without the methoxy group. Moreover, insertion of the substituent is expensive and so you have a less active but more expensive molecule. The alternative approach is to insert in the side chain a syn-oxime. This route was first taken by Glaxo who developed Cefuroxime. Molecules, in general, with this alpha methoxime substituent, tend to show β-lactamase insensitivity. The compounds are not as insensitive as with the alpha methoxy substituent. But, nevertheless, for clinical purposes, it is adequate. The gap in the lactamase resistance problem which perhaps is important and which people argue about is that the enzymes of Gram-negative anaerobes such as *B. fragilis*, still have the capability of hydrolysing the cephems which have the oxime substituent. But the upshot of all this was the emergence of resistant *Staph. aureus*, the emergence of resistance in Gram-negatives and the development programmes that went ahead so that one has Ampicillin and all those early penicillins whose position is being eroded because of the emergence of resistant strains. We have a string of new cephalosporins, of which the latest that is coming into the market in Germany tomorrow is Cefotaxime, which are really good, really effective molecules and with β-lactamase resistance. And then the question arises again, haven't we gone far enough? Why don't we stop at this point? What is wrong with what we already have and why do we need anything more? I think it is really important to look at what may happen in the future. Some of the following questions can be asked. We want in our antibiotics for the future, activity, of course. We want resistance to β-lactamases. This we have achieved somewhat by the methoxy substituent or methoxime substituent. We have devised cephem molecules which are resistant to most β-lactamases. We want to have compounds which can be orally administered. One of the big problems that we have heard is that it is extremely difficult to devise active, β-lactamase resistant, orally absorbed cephalosporin derivatives. If, indeed, we could find a good, oral cephalosporin, really β-lactamase resistant, then this would be a major advance. There are almost certainly possibilities for having advances with respect to pharmacokinetics.

Many of the molecules, particularly the oral molecules that are available, have a rather short half life, and instead of injecting an antibiotic into someone 3 times a day, it would clearly be an advantage, both economically and for the comfort of the patient, to inject once a day. So there is a certain pressure on people to design compounds with a longer half life and, therefore, a greater convenience of administration. Certain of these new molecules that we are looking at, at the moment, are very effective, they are β-lactamase resistant but they have, perhaps, and this remains to be evaluated in the clinic, the possibility that their bactericidal property, their absolute knock-down properties against bacteria are not quite as sharp as they could be. But dominating the whole of the situation, I believe, is the question of cost because these molecules, very active, very enzyme resistant, such as Cefotaxime, Cefuroxime and even the newer ones which are scheduled in the future will be very expensive. And we cannot escape the fact that in all societies in the world, the question of payment for medication is coming under tighter and tighter scrutiny by government. The question of cost must become the dominant one in the whole future of β-lactam antibiotics. It is not any good, in my opinion, to go on designing more and more sophisticated molecules and having them ever more expensive. Because, ultimately, you get a wonderful drug which no one can afford.

I think I would like to go on now to the second part of my talk to first stress the points where I think there may be shortcomings in the currently highly developed β-lactams. And then, perhaps, outline for you where I think there are chances in the future, especially those dominated by cost and economy. Now if one looks at the bacteria under the action of the β-lactams, one finds the emergence of structurally altered bacteria as intermediates in the killing process. In the presence of certain β-lactam antibiotics and, for example, Cefuroxime is rather characteristic in this respect, one rather easily generates long forms of *E. coli* at concentrations which are rather below the M.I.C.'s. Now the reason for this is, contrary to what was believed for a long time, when β-lactam antibiotics act on bacteria, they attack them in a variety of different ways. There are certain targets in the bacteria which are basically targets that lead to lysis and there are other targets in the bacteria which lead to elongation. The elongation is because the cross septum formation in the bacteria has been inhibited. There are other targets which produce very big bloated forms of bacteria. And over the past few years, people have been developing means of looking to see the extent to which one can analyse the binding and the attack of β-lactam antibiotics on various aspects of cell-wall synthesis. Now to do this one tries to measure the binding of β-lactam antibiotics to the various targets in the bacterial cell and these various targets can be visualised by making preparation of the membranes of the bacterial cell and spreading them out in isoelectric focusing gels after denaturing the proteins. And when you do this you find in Gram-negative cells like *E. coli* six or seven proteins which bind β-lactam antibiotics. This technique was developed by Strominger and by Brian.

What you see, when you have isolated the membranes, bound benzylpenicillin to them and then separated them in the isoelectric gel is something that is recognized diagramatically as shown in Figure 4.

Penicillin binding proteins and the proportion of benzyl-penicillin bound in preparations from E. coli.



Data from Spratt (1977)

Fig. 4

You have various penicillin binding proteins as they are called. In fact, the majority of the benzylpencillins are bound to two proteins which run fast, that is, they are of low molecular weights. About 85% is bound to these two. What is interesting is that some of these binding proteins have been identified as enzymes that are related to various aspects of cell wall synthesis and, therefore, if one binds antibiotics to various of these enzymes and if one does it to differential extents, then one produces different effects during the subsequent growth of the organisms. Therefore, if one has an antibiotic that binds very strongly to protein Ib, you have an antibiotic like Cephaloridine that produces lysis. If you have an antibiotic that binds very strongly to protein III, you have a compound that primarily produces long forms of the Gram-negative organisms and does not kill them immediately. If you have an antibiotic that binds protein II, you have a compound which forms large bloated forms and the bacteria do not die immediately. Figure 5 shows you the relative bindings to the different proteins.

Binding concentrations of some β-lactam antibiotics
to E. coli penicillin binding proteins

	Antibiotics					
PBP's	CER	CEZ	CET	CXM	HR756	FK749
Ia	0.25	0.2	0.2	0.1	0.02	0.02
Ib	2	5	16	2	0.7	0.6
II	6	5	37	32	5	50
III	8	6	1	0.05	0.05	0.01
IV	17	38	60	125	125	125
V	500	250	250	250	125	50
VI	500	250	250	250	125	250

All values µg/ml.

CER.. Cephaloridine   CEZ.. Cephazolin
CET.. Cephalothin   CXM.. Cefuroxime

Fig. 5

If you look at the relative binding of different β-lactam antibiotics to these proteins, one begins to see rather different effects. Cephaloridine, for example, has a certain amount of binding to protein 3 and at 8 mcg/ml, Cephaloridine will produce long forms.

Now, if you look at some of the newer compounds such as Cefuroxime, you find that it has somewhat similar binding properties as Cephaloridine, as far as lysis is concerned. But it has a very, very low level. What I mean is that a very small amount of Cefuroxime is required to inhibit this enzyme that causes the formation of long forms, when inhibited. If you take Gram-negative bacteria and treat them with low concentrations of Cefuroximes you very easily produce long forms. And it is only at rather higher concentrations that you get lysis. And the same is true, but to a lesser extent, for HR 756 or Cefotaxime, and with Fujisawa compound FK 749. So, these are β-lactam compounds which are certainly very effective but they perhaps do not hit with their full weight or they would in fact be more bactericidal in my opinion, were they to have a sharper effect on this penicillin binding protein Ib. So we have, in the latest molecules to be developed, certainly major advances; but if you are looking for one aspect in which they can be improved, they could be improved so that they produced more complete and total lysis. This is not to say that you can't easily get 0.7 mcg/ml. in the circulation. With Cefotaxime you can, but at the limiting concentration you tend to get long forms. So that is perhaps a minor disadvantage of what is, otherwise, a major advance. And this minor disadvantage is shared both by Cefuroxime and FK 749. Another area where one has problems is the area of oral absorption, and many of the orally administered cephalosporins have a binding primarily to protein 3 and Cefalexin, for example, is an antibiotic which one always sees as producing long forms rather easily. So one other shortcoming of the present series of highly developed cephalosporins is that we do not have a really active, really β-lactamase resistant, oral cephalosporin.

To revert to the question of penicillin. People have not managed to produce derivatives of penicillin that are resistant to β-lactamase hydrolysis and a certain amount of virtue has been made of that fact because what people have started to do now is to look for molecules that are β-lactamase inhibitors. The idea being to add these inhibitors to old antibiotics to enhance their activity with resistant strains. The inhibitor would inhibit the β-lactamase and leave the old antibiotic free to do its job like it used to do. Now the attraction of that approach is that if you can get an inhibitor that is really cheap and effective you can add it to an antibiotic which itself is already cheap and effective and if you are lucky it will be orally administered and, therefore, you will come forward with many of the advantages of the latest cephalosporins but with additional advantage of oral activity and cheapness. So

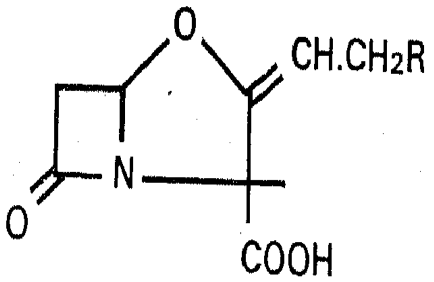


Fig. 6

there are powerful clinical and economical reasons for looking for such inhibitors. And the discovery as a natural fermentation product of Clavulanic acid, a molecule of the general structure shown in Figure 6 illustrates the point.

There is nothing in the 6 position, oxygen in place of sulphur and with a variety of different substituents. For example, a molecule with a methyl which is a natural fermentation product. These molecules have turned out to be very potent inhibitors of some β-lactamases. Now, that is a very big advantage, and it would be a superlative advantage if they were inhibitors of all types of β-lactamases, but they are not. They are inhibitors of some important ones, but not the whole range of β-lactamases. So one is left with the fact that this molecule is an inhibitor which is quite interesting. It has the advantage of being a primary fermentation product which will be quite cheap. It will support antibiotics that are themselves cheap, it is orally active but its disadvantage is going to be that if you want to broaden the inhibitory spectrum in order to cover all the β-lactamases you are going to have to involve yourself with semi-synthetic chemistry and you will then be back with the problem of having an expensive antibiotic because this will be an expensive addition to a cheap antibiotic and one will say that if you want to support a cheap antibiotic you should not use an expensive compound to do it. Clavulanic acid is, therefore, an interesting compound though it has a limited range and it will be interesting to see whether a combination of Clavulanic acid with Ampicillin or Amoxicillin will have any impact. The advantage is that they may be a good deal cheaper than the latest cephalosporins. Another primary fermentation product which has emerged, which is interesting in this context, is a group of compounds called Olivanic acids (Figure 7).

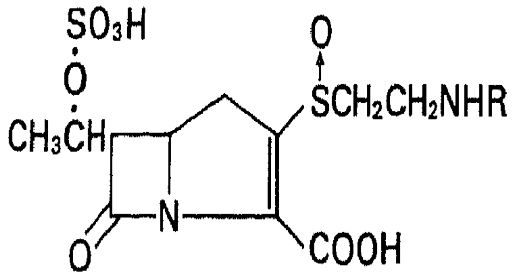


Fig. 7

These molecules are very often oxidised on the sulphur and carry a sulphonic acid, compounds very similar to Thienamycin which has a double bond in the ring and carbon in the place of sulphur and a side chain in the 6 position. These molecules are also potent β-lactamase inhibitors and can be added to Amoxicillin to support that molecule in the presence of resistant organisms but again there is the disadvantage that these molecules are not broad spectrum inhibitors.

Another approach has been to use pure organic chemistry to design inhibitors. This is shown in Figure 8.

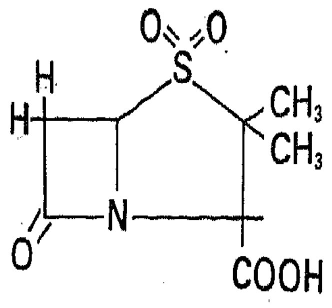


Fig. 8

The Pfizer company has produced this particular derivative based on the penicillin nucleus, very cheap indeed and very simple, with the sulphur oxidised. Unfortunately, this is not a very good inhibitor because this β-lactam bond is susceptible to hydrolysis by β-lactamases. Therefore, although the β-lactamase is inhibited it is not inhibited to the extent that it will not destroy the inhibitor. Most people feel that this very cheap inhibitor will not adequately support Ampicillin or Amoxicillin. Moreover, this suffers from the same disadvantage as Clavulanic acid and Olivanic acid in its limited range of inhibitory property. Other synthetic compounds that have started to emerge are shown in the next figure (Figure 9), where you have a bromine or chlorine or iodine in position 6 of the β-lactam ring of the penam structure and no oxidation of the sulphur.

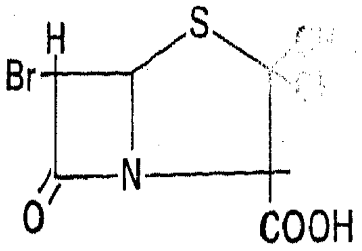


Fig. 9

In general, these molecules tend to be better inhibitors because they are irreversible inhibitors of the enzymes. The enzymes cannot hydrolyse them and, therefore, their effects are more long lasting. Again, cheap molecules which you would add to a cheap antibiotic but with the same disadvantage of a limited range of inhibitory property. So one can summarise the present situation by saying that we have highly sophisticated, very resistant, injectible cephalosporins which have many of the properties that we want. We have very cheap penicillins that need supporting by the addition of extra molecules to make them active against resistant organisms. The penicillins have the advantage that they are oral while the cephalosporins are not. So we have still not reached the ultimate situation which I would believe would be a β-lactamase resistant, broad spectrum oral cephalosporin. Basically, the approach that has been used to achieve the above objective has been esterification (Figure 10).

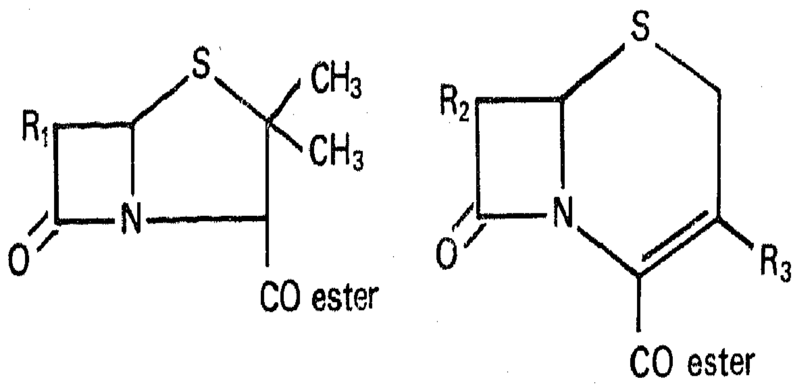


Fig. 10

In the penicillin series that produces molecules that have enhanced oral absorption. In the cephalosporin series, nothing has appeared in the literature so far to suggest that equivalent esters in the cephalosporins will be oral molecules. But in some places we begin to hear rumours that some esterified derivative of the latest cephalosporins are beginning to look interesting, to have oral absorption high enough to be useful and, therefore, I think there is a chance and, if I have to bet, I will bet that there may well be some oral esters of the most sophisticated cephalosporins in the market within the next 5 years. It is only a guess, and in which case some of the advantages which still remain with the penicillin series may get eroded. But the oral cephalosporins are going to be expensive because the cephalosporins that are being made into oral compounds by esterification are themselves expensive. And so if I am dominating with this theme of cost, and I think it is important, and I think it is really the provision of a molecule that has all the properties that one wants and is cheap which is the real prize to be won in this field. Dr. Coombes mentioned to you the so-called penem series. This is a group of molecules which have a double bond in the 5-membered ring, sulphur, like penicillin, but because of the double bond, only one substitution possible (Figure 11).

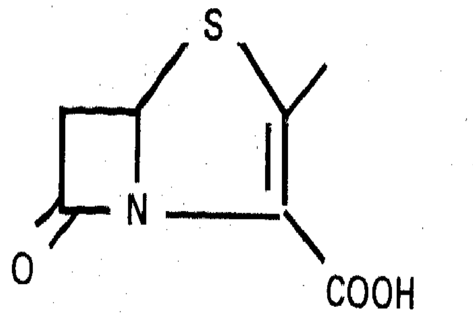


Fig. 11

And the question now arises as to whether by organic chemistry, as opposed to fermentation, people cannot make derivatives which are rather simple and which have all the properties that we desire. The enormous advance that has come, in my opinion, over the last few years is the realisation from the Thienamycin series that you can have very potent β-lactam antibiotics without a sulphur in position one (Figure 12).

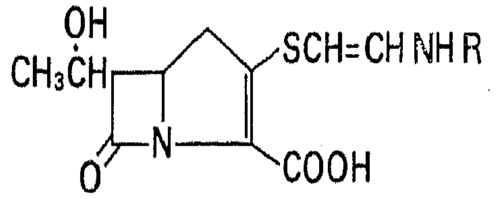


Fig. 12

Because, as soon as you remove the ring sulphur, you have a molecule which is much easier to synthesize by pure organic chemistry. And my guess is that the future oral β-lactam antibiotic is going to be a derivative with a rather simple substitution such as a methyl or an ethyl group and with something which will give it some cephalosporin-like properties, a carbamate, for instance, and we may find that such a molecule has a combination of properties that has some of the advantages of the penicillin series and some of the advantages of the cephalosporin series. And I think the most interesting thing is that, suddenly, by the realisation that organic chemistry may be able to solve some of these problems, one has opened up the field in a very wide way for very many people to get into it. Because we already know that the derivatives of the penem series are active as antibiotics and, therefore, roulette can be played on that structure.

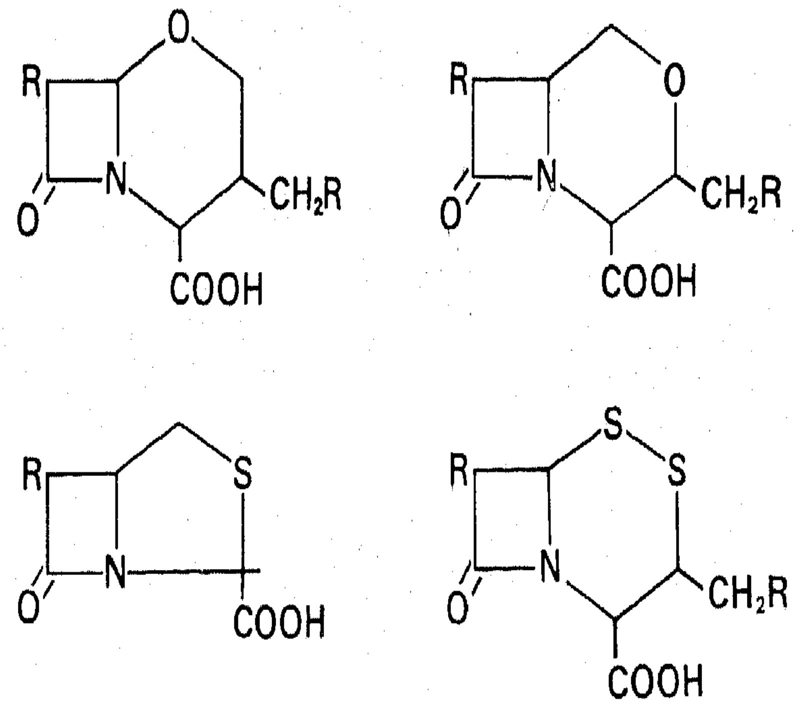


Fig. 13

We also know for example from the patent literature that the cephalosporins with an oxygen in the ring are active (Fig. 13).

We know that you don't have to have the oxygen in position 1 necessarily but it can also be elsewhere. We know that a disulphide in the ring is active. We know that the sulphur in position 2 is also active. They are not very active in that form but they are all potential starting points and since you don't necessarily need big substituents at that point and certainly not amine derivatives or peptide derivatives one has the great possibility for organic chemistry to lead to molecules that are orally absorbed, highly active, cheap and resistant to β-lactamases, which is a combination of properties that we don't yet have in any one compound but we do have it in a wide range of individual compounds combined together. So, a general feeling is that the recent discoveries in the novel nuclei are giving people a clear idea as to where to go to in organic chemistry and I think that the β-lactams of the future may well be the products of the organic chemistry laboratory which I think is a sad reflection on the original fermentation routes that were used all those years back. But I suppose one is left with the abiding feeling which, I hope I have made clear and which I leave to you to contemplate, is that more is not always better.

PANEL DISCUSSION

Topic: **Problems encountered in antibiotic therapy**
Chairman: Prof. E. v. Wasielewski

Members of the Panel: Prof. M. Richmond, Dr. K. G. Nair, Prof. O. Siddiqi, Prof. C. K. Deshpande, and Prof. H. Zaehner.

From the left : Prof. Siddiqi, Prof. Deshpande, Dr. Nair, Prof. v. Wasielewski, Prof. Richmond and Prof. Zaehner.

Prof. v. Wasielewski

The last part of this interesting symposium is a panel discussion on the topic "Problems encountered in antibiotic therapy".

Two remarks first. "Problems encountered in antibiotic therapy" is a theme which is not to be covered in 2 or 3 weeks. So we can only pinpoint some problems; and as you well know from the afternoon and the morning talks it is funny that actually we did not discuss, with the exception of one speaker, and I mean Prof. Zaehner, we did not really discuss antibiotics. We discussed β-lactam antibiotics and this has a reason may be and I hope we will come to that point later. So we can discuss, the audience and the panel, only a small section of the antibiotic field. Before we start, I may very briefly give you an idea of what we are doing, what we are planning, and what are our objectives or goals. What we have to cover really is first the chemistry and the aim is to have a compound whose chemistry is achievable with normal methods. This was the point Prof. Richmond mentioned in the afternoon. It must not be so costly that you cannot sell it, nobody can sell it.

Microbiologically, it should have a broad spectrum, it should have high M.I.C. values, it should be stable against resistance mechanisms of bacteria. It should not induce resistance or cross resistance. It should have no or very little effect on intestinal or other local flora. Those are ideal objectives or those which we hope to achieve.

The third point is the pharmacology and toxicology. The compound should have an optimal therapeutic index, which means no organ toxicity, no allergic potential, no influence on the immune system, no embryo toxicity and no teratogenicity. Furthermore, no neo-natal/or young child toxicity.

It should have really an optimal pharmacokinetic. It should show a good intestinal absorption. A good local tolerance and a good distribution in tissues, particularly around the infection site. It should not have a high protein binding, it should not be metabolised, it should be excreted as an intact compound and not as a metabolite or a variety of metabolites. It should have a half life span which is adequate to its usage. Once a day applicability is desirable. That means that it should have a good galenic, i.e., the properties to make the needed formulation must be in the chemistry. A chemical, stable in different formulations, orally and parenterally applicable and compatible with other drugs. This was only the preamble to our panel discussions.

Prof. v. Wasielewski

Prof. Zaehner, Do we need new antibiotics? You have already answered this question in your talk. Would you very briefly repeat why?

Prof. Zaehner

One reason is the appearance of resistant strains. Secondly, there are many fields for antibiotic application that are not yet covered by good antibiotics, for example, infections with viruses, protozoa, mycosis, etc. Even outside the medical field, the area of plant protection is still to be covered.

Prof. v. Wasielewski

Prof. Richmond, this morning Dr. Coombes spoke of classical and non-classical β-lactams. Do you feel that the future lies only with these β-lactam antibiotics? Should we forget the tetracyclines, the macrolides, the lincomycins, aminoglycosides, etc.?

Prof. Richmond

I think the enormous advantage of the β-lactams is their very favourable toxicology. If one can obtain molecules which are really active in this series then we have reasonable hope, because of the very favourable therapeutic index, that we will have effective molecules. One does not really forget the other classes of antibiotics but I believe that β-lactams are our best bet.

Prof. v. Wasielewski

Prof. Deshpande, do you agree with this opinion?

Prof. Deshpande

I would like to say that we in India have to face many problems that are different from those of the western world. We have many bacterial diseases which still pose a number of problems. For instance, leprosy is no problem to you but it is a very big problem to us. In our opinion we think that a real breakthrough drug is still to come. We look forward to the pharmaceutical chemist to give us the answer. In a way it is true of quite a few other infections that have been eliminated in the developed world but which are still with us and need to be attacked on all fronts and, therefore, I feel that there is room for many more antibiotics and effective ones.

Dr. Nair

I agree with Prof. Deshpande's comments. This morning a lot of stress has been laid on β-lactams, and rightly so, but here as a practising physician, I find that there are 2 million cases of leprosy in India and the treatment is far from satisfactory. We see in India the problem of resistant Salmonella infections. We need a very good drug against Salmonellosis, especially against typhoid fever. We have the problem of severe infections with resistant pseudomonas and most of the general practitioners in India are still looking for an oral, non-allergenic penicillin. They are afraid to give injections. And this is still the desire of those practising medicine.

Prof. v. Wasielewski

This morning Dr. Coombes, Dr. Richmond, Dr. Schrinner have all talked of β-lactam antibiotics and their very favourable properties, especially toxicology. However, there is still the problem of allergic reactions and resistance as we have seen. I would like the audience to comment on this problem.

Dr. Schrinner: I would like to ask Dr. Nair whether he believes that the oral β-lactams are less allergic than the parenteral ones.

Dr. Nair: I don't believe that they are less allergic. But, the incidence of severe anaphylactic shock following a penicillin injection is still very much in the mind of the medical doctor, in India particularly. I am just suggesting that you may be able to combine, not today but five years from now, some molecule which will counteract the IGE mediated reaction with penicillin and then provide something orally. This is possible perhaps as research is going on in this area.

Prof. Richmond

I am very interested in this worry about the anaphylactic reaction. I mean, is it something which is very characteristic of β-lactam usage in this country? Because, if you talk to general practitioners in Britain who use enormous amounts of β-lactams, then they may see hardly one case of anaphylactic reaction in their whole medical life time. I mean, is it really a problem?

Dr. Nair: Yes, it is a problem and it may depend on the purification and properties of the penicillin compound. There are people here from the industry, Hindustan Antibiotics for instance, who may like to answer this question. It is a very real problem.

Prof. v. Wasielewski: The Beecham group carried out a lot of work on this aspect of the problem and they concluded that the purity of the penicillin was not a factor in the allergic reaction. The basic molecule itself is responsible. The β-lactam ring, the thiazolidine ring and the side chain are three groups which may give rise to allergic reactions in the human system. Is this allergic problem really going to change the picture completely regarding the use of β-lactam antibiotics?

Prof. Deshpande: In India, general practitioners have really crowded clinics. If they administer a penicillin injection which can lead to a anaphylactic shock, then they have a problem in handling this case in the midst of their crowded schedule. They, therefore, tend to avoid taking the risk of administering an injection and would rather go for an oral compound which they believe would be less toxic. Another reason for the paucity of actual figures of such reactions is the tendency in many cases to hide such reactions and attribute it to other factors.

Prof. v. Wasielewski: I would agree with Dr. Deshpande. The problem is the same all over the world. Even in the U.S., around 300 cases of reactions have been reported in a year, and this does not cover the reactions with side effects such as rashes, uriticaria, bronchospasms, etc. A lot of people are trying to tackle this problem. We are looking for conjugates which one can use in order to prevent an increase of allergy, in IGE antibodies or, if you have an allergic state already, to block the antibodies. But the problem is really that you have to give this blocking hapten in the ratio of 1 : 1 and this makes a big bulky pill that you have to give. A second problem which I would like to discuss is the problem of resistant strains. I would like to ask Prof. Siddiqi to comment on this problem.

Prof. Siddiqi

The main problem with resistant strains is that the transfer efficiencies are very high. Most resistant strains when they arise, especially when they arise by mutation, if transfers were not so efficient, then these would tend to disappear by themselves. All transfer involves some surface property of the recipients. It should be possible to block transfer by having additional treatments. This would basically mean treatment with drugs or things which are aimed at blocking surface properties which are essential for conjugal transfer. I think it is very difficult to conceive that all resistance transfer factors can be eliminated. There are too many of them and they all arise in a natural context and bacteria have to have them. Even if you eliminate them, they will reappear. But I would assume that something can be done to block transfers in particular cases.

Prof. v. Wasielewski: Prof. Richmond, would you comment on this please?

Prof. Richmond: I am rather pessimistic about designing molecules to block transfers. It is not really difficult. There are many detergents that are already available and even some compounds that are used quite widely in clinical medicine will block transfer extremely efficiently. But all the patterns that one sees suggest that transfer is not important during the course of a particular condition. The transfer is essentially a strategic phenomenon and it may be occurring in people who are not ill and, therefore, that argues that if you are going to have an agent that will block transfer you have to treat the whole of the population continuously with these agents and this does bear thinking about.

Prof. v. Wasielewski: Dr. Nair, do you think that resistant strains are really a problem or can you escape by choosing another antibiotic?

Dr. Nair: The answer is yes. There are real possibilities. When Gentamicin was introduced 3 years ago, it was claimed that there was no resistance possible in that compound. We now have Gentamicin resistant strains, Salmonella resistant to Chloramphenicol, we have many urinary tract infections that are recalcitrant and the worst ones we see are the resistant pseudomonas infections. We hope some of the new antibiotics that are being released will alleviate the problem at least for some time. However, physicians have a tendency, at least in our country, to grab at the latest compound on the market. I think that there is a need for an antibiotic policy as you already have in your country and in Finland, whereby the new antibiotics are made available only to professors of medicine and microbiologists. They are not available to the general public except in cases where it has been examined by a committee.

Prof. v. Wasielewski: I am always a bit puzzled. Is the problem of resistant strains not a problem of hospitals more or less or of communities and towns and what is the opinion of the practitioners?

Prof. Deshpande: I think you have really highlighted the point. The incidence of resistance within the hospitals is as alarming here as in the West. It is more alarming here because we have not only indiscriminate use but also irrational use. You may advice a person to take so much of a dosage for a period of time. It may not necessarily be followed for many reasons, including the educational factor. Outside, I would agree that the incidence may not be that alarming, again for the very simple reason that very few can afford to use expensive drugs. In our situation the hospitals provide most of the drugs free, including Gentamicin, and that creates a real difference. So in our setting, this particular differential is very important. But the point is well made that hospital infections go hand in hand with the special problem of resistant strains emerging and they may remain for quite some time and a remedy suggested should be practicable here also and sooner or later we have to take a serious note of the fact that we have to have a reserve antibiotic for every possible problem bug.

Prof. v. Wasielewski: I think it is very interesting that there are very many strains that did not become resistant during the years of antibiotic therapy. The streptococci, for instance, do they have plasmids?

Prof. Richmond: Yes, they do have plasmids. Tetracycline resistance, for instance, in group 0 streptococci. It is very dangerous to be too complacent though, because the pneumococci, for example, for many, many years were very sensitive to penicillins and quite suddenly, about 2 years ago in South Africa, there was an enormous outbreak of penicillin resistant pneumococci. This happened almost 30 years after the introduction of Penicillin and the same is true for gonococcus and hemophilus.

Prof. v. Wasielewski: I would like to ask Prof. Zaehner a question. In the morning you presented a chapter on new recognition methods and, if I understood you correctly, you said that we can screen and we should screen also old strains and may be they show a new activity which we can use and which we are missing when we don't use this method. Can you elaborate on this?

Prof. Zaehner: In the beginning many strains were screened very rapidly and superficially. We should now look more carefully at some of the old antibiotics, look at their mode of action, their mode of transport. I cited this morning the example of Pleuromutilin. This is an old antibiotic. But it has now been possible to make derivatives which are much more active and can be marketed. I don't know how many of the old antibiotics can be derivatised in this way but I think it is worth looking into.

Prof. v. Wasielewski: I think that is a very interesting point. Though we have about 40 clinically useful antibiotics, only about 5-6 are in general practice. We should certainly look for new activities. May be Dr. Nesemann or Dr. Divekar can comment on this.

Dr. Nesemann: I feel that the present level of antibacterial activity that has been reached with compounds like Cefotaxime is so high that it may be impossible to directly get new antibiotics with superior properties. We have to look for new structures and develop these chemically to get new antibiotics with new qualities. Pleuromutilin is a good example and I feel we should proceed in a similar manner with other antibiotics.

Dr. Divekar: I can only add two examples. Mycophenolic acid is a very old antibiotic but we are now finding an use for it. Grisefulvin is another old antibiotic which was originally supposed to be only a curling factor but has found clinical use only after several years.

Prof. v. Wasielewski: Prof. Siddiqi, is it possible from the angle of genetic engineering, to find new structures or construct new structures by transferring a set of genes from one antibiotic producer to another?

Prof. Siddiqi: To conceive of such a possibility, you have to use your chemical intuition. You have to have an idea of what you want to make. From the point of view of genetic engineering, I don't see any difficulty. Once you have a set of enzymes which catalyse a sequence of reactions you can put them on a vehicle and introduce them into a new cell or even perhaps a new chromosome. But to conceive of something which does not exist and which you want to make, I think it is a different matter altogether. I think it is for chemists and pharmacologists to say what is it that they might make which we cannot think of at the moment.

Prof. Richmond: I would argue that we are very near the position where we do not want any more human therapeutic antibiotics for bacterial infection. And I think I would argue that what we do need are probably therapeutic agents or antibiotics or man-made molecules for a variety of conditions which at the moment are regarded by the pharmaceutical industry as uneconomic targets, e.g. Malaria.

Prof. v. Wasielewski: This is partially true but not completely true. A lot of work is being done on malaria from the immunological side and also from the chemotherapeutic side. But it is extremely difficult to find compounds that are better and there is, as far as I know, no compound from the antibiotic side which is active so far in malaria. I would like to come back to this topic later. Now, I would like to ask our 2 clinicians, what is their desire from the clinics for new antibiotics. We just learnt from the presentation of Dr. Schrinner the very interesting fact that from 1935 to 1947, the mortality rate from infectious diseases dropped. But, from 1947 upto today, the mortality rate has remained static. Undoubtedly, the cause of the mortality changed from acute diseases to chronic diseases such as bronchitis or urinary tract infections. So, I would like to ask you what are the wishes of the clinicians. Is it for better pharmacokinetics or for an absolute better antibacterial activity or for a compound with less side effects.

Dr. Nair: At least for the third world, we would like a broad spectrum antibiotic either from the penicillin field or from the cephalosporin field which can be orally administered and, preferably, it should be inexpensive and with minimum side effects. I may say in passing here that when we are talking of side effects and such we may be the victims of this medical nemesis where, by throwing these things around freely, we may not be giving the immune system of the patient a chance to respond to disease at all.

Prof. Deshpande: I don't agree fully with the thesis proposed by Finland. In terms of epidemiology and statistics, total infectious diseases as a whole, I would accept. But, to me, it appears as though you have eliminated one problem and in the process you have created another. For instance, you are treating a person with an antibiotic which is going to kill a lot and with corticosteroids, then you are making room for an opportunistic infection. And when this opportunistic infection occurs you find that you really did not have an antibiotic to meet that. Then you go for another antibiotic which would kill that also effectively. So this pattern is changing. Also there was an assumption before that certain organisms did not cause much problems, such as anaerobes. We were not aware of these till we actually came across situations where you could show, for example, with Metronidazole that you could meet some of the problems of bacteroides infections. So, broadly speaking, I may agree with these statistics. But when you analyse we do feel that in eliminating certain things we have created biological vacuums that have been filled by certain other agents and that is how the problem appears to be perpetual.

Question (Audience):

I would like to add another property to the list of properties of an ideal antibiotic. The antibiotic should be a compound that is target specific, i.e., it should go directly to the site of infection and not bathe the whole system. It should be active only against that particular infecting organism. This would solve the problem of resistance and so on.

Prof. v. Wasielewski: That would mean a very narrow spectrum antibiotic active against a specific organism only and leaving the rest of the flora intact. This is a desire but very difficult to achieve.

Question (Audience)

Mr. Chairman, in your introduction you stated that an ideal antibiotic should have a low protein binding. I really question that, because we could have a β-lactam antibiotic with a high protein binding and yet be quite effective. Is the binding that essential? Or could it be that what is important is that you don't have an irreversible protein binding?

Prof. v. Wasielewski: Dr. Schrinner, would you like to answer this question?

Dr. Schrinner: I really did not want to step into this. The controversy is still going on. I also do not agree with you that we need a low protein binding. Certainly, protein binding (to albumin) plays an important part in pharmacokinetics. The question really is how tight is the binding and is it reversible? In areas of inflammation we get an increase in albumin and, therefore, binding to albumin may be a positive factor but it must be reversibly bound. The question is complex.

The binding must be reversible and to measure the tightness of this binding is not possible as yet. One can always use a salesmen's approach and say that when there is no protein binding it is an ideal compound because the free antibiotic is required for activity. On the other hand, when there is protein binding, we can say we have a compound which has a much better half life and prolonged activity. Therefore, to discuss protein binding in this way is futile.

Prof. v. Wasielewski: This is certainly true. Are there some more questions? Then we could revert to the topic of the influence of the immune system.

Dr. Divekar: If I may be allowed a question. With the intensive use of β-lactams is there any possibility of breeding super β-lactamases? I think Prof. Richmond or Prof. Zaehner may be able to throw some light on this.

Prof. Zaehner: I have my own opinion in this field. Perhaps, it is unfair to say that without the use of antibiotics we would not have a resistant problem. We should save antibiotics as much as possible. We should not misuse antibiotics by taking antibiotics in every case without necessity.

Prof. Richmond: I will simply state the belief that is prevalent. I think that β-lactamases as we see them now are actually extremely ancient inventions and the amount of evolution one sees, primary evolution to entirely novel β-lactamases, is very small. The evidence is very limited. People have looked back, for instance, in collections of soil made 300-400 years ago and they have found spores in the soil samples and they have isolated the bacillus species and they have looked at the β-lactamase and they have found it to be identical to the enzyme we now know. There

is the classic case of the New Zealand hedgehog. There are hedgehogs in N.Z. which very commonly have skin lesions caused by dermatophytes which produce β-lactam antibiotics and these lesions are infected by *S. aureus* and if you look at this *S. aureus* it makes penicillinase and that penicillinase is identical to the one which we now know causes clinical problems in man. So I think β-lactamases are very ancient inventions and the chances that one would see spectacular new inventions is very, very limited. So what one can say is that what one is seeing is a selection from the pool which already exists and at a certain point this will possibly come to an end.

Prof. v. Wasielewski: I think we have to answer the question of influencing the immune system. The question has two parts. One part is: do antibiotics influence the immune system? The second part is: when we use antibiotics in any infection, does the immune system become untrained? Is that what you meant?

Audience: No, what I meant was that, in the West, most of the diseases have been conquered by better public health procedures rather than by antibiotics. In a country like ours, should we not also emphasize the public health aspect much more? Secondly, when one gets an infection and we go to a clinic the doctors administer immediately a broad spectrum antibiotic. This may result in the selection of resistant strains which we are talking about. What I mean is, why not give the immune system of the patient a chance to be effective, without using any antibiotics?

Prof. Richmond: As I have mentioned, in Britain from around the 1870's the improvement in acute infectious diseases has occurred more or less continuously. The major reason for this has been mainly due to the building of hospitals, better civil engineering, better patient care and handling, though therapy has also played a part.

Audience: I would like to comment on the evolution of certain new types of enzymes. In the Hudson River, scientists from G.E.C. found bacteria containing plasmids which code for enzymes capable of breaking down chlorinated hydrocarbons. These compounds were introduced only 30-40 years ago. Therefore, this must be a recent evolution.

Prof. Richmond: For this argument to be sound, you have to be sure that the enzyme on the plasmid cannot also metabolise some other compound and you cannot know that. Methicillin resistant staphylococcus was around long before Methicillin was ever invented. This is because these strains are also resistant to other β-lactams that were around earlier. Therefore, how can you be sure that the enzyme which metabolizes your compound does not also metabolize other compounds that have already been around.

Audience: Would you, therefore, say that most of these plasmid borne genes must have evolved a long time ago or evolved very slowly and, therefore, one need not worry about the evolution of any new genes coding for enzymes...........

Prof. Richmond: No, I am not saying that evolution does not occur. I am only saying that the time scale is slow. There may possibly have been an evolution recently but what I am saying is that, most probably, these enzymes have been around for a very long time.

Audience: Is that being said only on the evidence of β-lactamases existing in the soil and which are only a few hundred years old?

Prof. Richmond: β-lactamases have evolved from carboxypeptidases that are involved in cell wall synthesis. And I believe that this happened a very long time ago. The evidence for that is not good. But evidence for protein stability is quite good. Life forms appeared a million years ago.

Prof. Siddiqi: I certainly think that the evolution of the ability to hydrolyse compounds cannot be looked upon as simply the evolution of one enzyme. It is almost certain that the ensemble of enzymes that have been found in the Hudson River bacteria have not been present in earlier isolates. So it is clear that once you create a condition that is highly selective you can create hydrolytic ability in an ensemble of genes which are present. Therefore, it is academic to discuss whether you modify an enzyme that already existed and was functioning rather poorly and now it does it efficiently because you alter something. But it is clear that bacteria do evolve and evolve fast because they grow much faster. Therefore, one cannot depend on the stability of bacteria too greatly.

Audience:

The more important point, I believe, is the time scale. Given a strong selection pressure they may be able to evolve and that too quite fast.

Prof. Richmond: I would like to make one point once again that if you take a new discovery, say, for example, a discovery of the transposon in *S. aureus*, you find that the same transposon was present in *S. aureus* which existed in 1930.

Audience: In that case, if you create a super β-lactam, would you say that there already exists some enzymes that will hydrolyse the super antibiotic?

Prof. Richmond: Yes, there certainly is a chance of that occurring.

Prof. v. Wasielewski: I think that was a wonderful discussion. I would like to now end this panel discussion by thanking the speakers and the audience for their lively participation.

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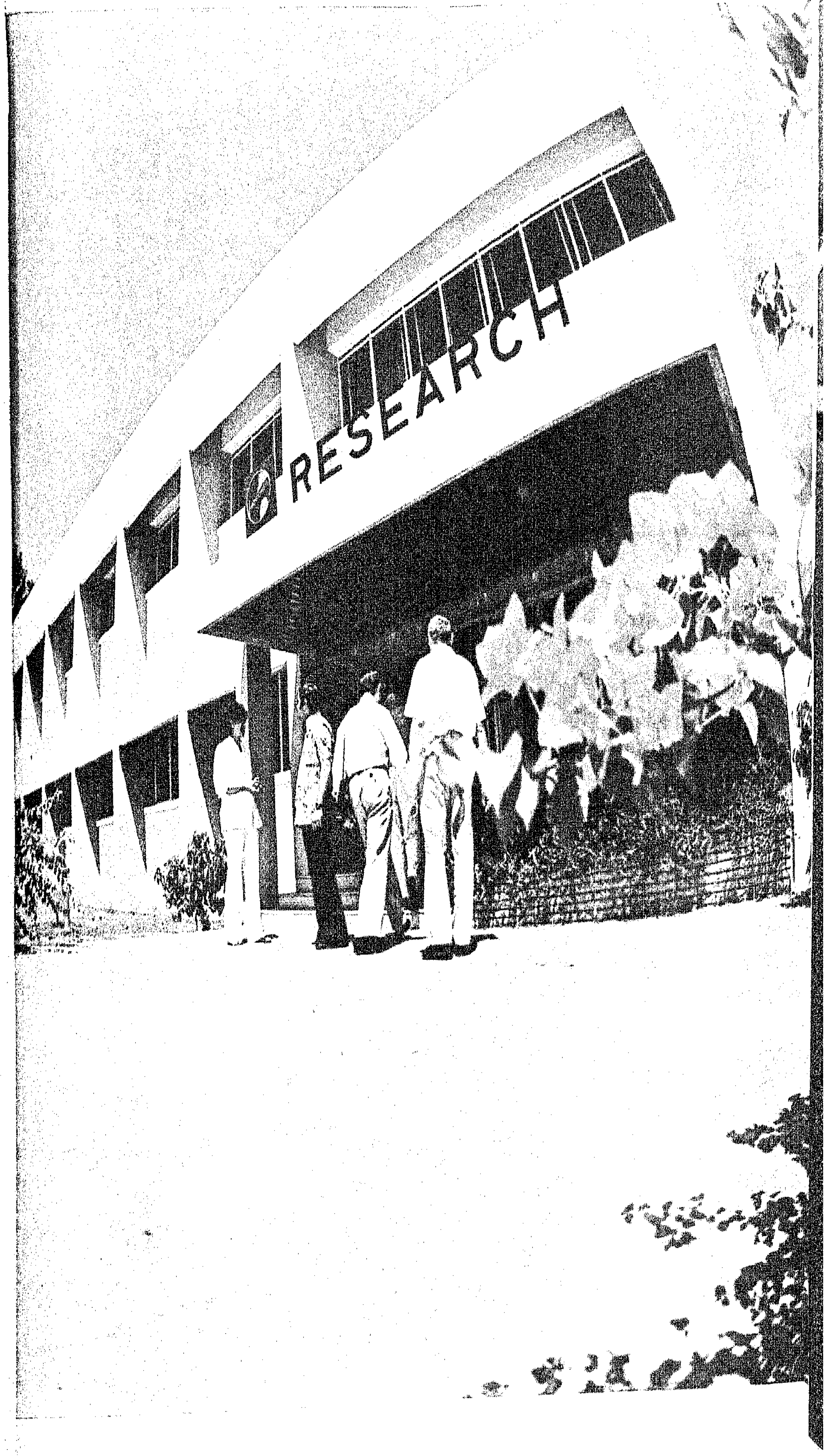GOA UNIVERSITY LIBRARY
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